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13. ABSTRACT (Maximum 200 Words) The general theme of this program deals with primary prevention and early diagnosis of ovarian cancer. We have identified several genetic changes in microscopic and early stage ovarian tumors tumor in Project 1, suggesting that these changes may represent early events during ovarian tumor development and progression. We have shown that Protease M over-expressed in a majority of stage I tumors and can be secreted by ovarian cancer cells in Project 2, suggesting that it is a potential marker for early detection of the disease. Furthermore, we have demonstrated that estradiol significantly simulated normal ovarian epithelial cell proliferation in Project 3, suggesting that there may be relationships between therapeutic hormone usage and ovarian cancer risk. Finally, we have identified new classes of lysophospholipids elevated in serum and ascites in Project 4, suggesting that these lipids can not only be used as diagnostic but also as potential prognostic markers for disease progression and novel therapeutic targets. Several new techniques have been established and applied. Collaboration among investigators has been initiated and several manuscripts and abstracts have been generated. Experiments have been finished according to the Statement of Work that we proposed in the proposal.					
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INTRODUCTION

Project 1: Early genetic changes in human epithelial ovarian tumors

Ovarian cancer is the fourth cause of death from all cancers among American women and ranks the highest among deaths from gynecologic malignancies. Although the cure rate with stage I ovarian cancer approaches 90%, two-third of patients are diagnosed with advanced intraperitoneal metastatic disease, with five year survival rate of 15 to 20%. Therefore, it is of paramount importance to identify a marker(s) for early diagnosis of the disease. However, it has been rare to identify Stage I disease and to see transition within a malignant tumor from benign to malignant epithelium which might help us to identify early genetic changes during ovarian cancer development. Recent histologic studies on prophylactic ovaries from high-risk individuals showed the presence of microscopic premalignant and malignant epithelia suggesting that they may create an identifiable milieu from which common epithelial tumors of the ovary will mostly likely arise. Molecular genetic study on these microscopic malignant epithelia would provide us with early genetic events during ovarian cancer development. We therefore propose first, to perform LOH study on specific loci on chromosome 1p, 3p, 5q, 6q, 7q, 9p, 11p, 11q, 12p, 12q, 14q, 17p, 17q, 22q and Xq by polymerase chain reaction (PCR) analysis of tandem repeat polymorphisms; second, to perform immunohistochemistry study on specific oncogene and tumor suppressor genes on paraffin sections prepared from ovaries with microscopic malignant serous lesions and to study specific oncogene activation and tumor suppressor gene inactivation by single strand polymorphism (SSCP) analysis and direct PCR sequencing on microdissected malignant serous epithelium obtained from paraffin-embedded ovaries; and third, to perform RNA fingerprinting on mRNA isolated from microdissected normal and malignant ovarian epithelial cells prepared from normal ovarian surface epithelium and early stage serous ovarian carcinoma and to identify differentially expressed genes in these early stage epithelial ovarian cancer cells. We believe that these studies should provide us with early genetic changes during ovarian cancer progression and serum markers which can be used for early diagnosis of the disease which will significantly improve the survival rate of the patient.

Project 2: A Potential Serum Marker for Ovarian Cancer

The poor prognosis of ovarian cancer is mainly due to the lack of sensitive tests for early detection of the disease, which is often asymptomatic. Studies have shown that ovarian cancer detected in early stage has a high five-year survival rate of exceeding 90%. Therefore, identification of molecular marker for early stage ovarian cancer detection is of paramount importance. This project is to study a cDNA sequence which we have recently identified by differential display. The encoded protein is highly homologous to trypsin and members of the kallikrein protease family. The novel protease, named as protease M, is highly expressed in many invasive epithelial ovarian cancer tissues and cell lines, but not in normal ovarian cell cultures. Since the preliminary data showed that upregulation of protease M was also observed in stage I tumors and the protease was detectable in the conditioned media culturing the tumor cells, the proposed work is to evaluate the potential use of protease M as a serum marker for early detection of ovarian cancer and for monitoring treatment response of ovarian cancer patients, similar to the use of another kallikrein member, prostate-specific antigen (PSA), in the diagnosis and prognosis of prostate cancer. The three objectives of this project are: 1) to study the expression level of protease M in normal human ovaries and ovarian tumors of different stages and histological grades; 2) to characterize protease M and to identify the physiological substrates for protease M by an innovative cyclic peptide library screening method; 3) to develop a sensitive, specific, and reproducible method for measuring the circulating protease M in the sera of ovarian cancer patients. The results of this study will have a

significant impact upon developing a substantially more efficient early detection program with an increased probability of reducing mortality from ovarian cancer. The characterization of protease M protein and identification of physiological substrates for protease M may provide insights into the probable function of this novel protease in the pathogenesis of ovarian cancer. The identified optimal peptide substrates with high specificity and affinity for protease M will have significant value in the development of a carrier for targeted delivery of cytotoxic agents to protease M-secreting ovarian cancer cells.

Project 3: Hormones as etiological factors of ovarian carcinogenesis

Ovarian cancer (OC) is the highest-ranking cause of death from gynecological cancers among American women. All cell types of the human ovary may undergo neoplastic transformation; the vast majority (80-90%) of malignant tumors are derived from the single layer of epithelial cells covering the ovarian surface. Although the etiology of OC is still unknown, several theories have been put forth to explain epidemiologic correlates. Nulliparity, lower number of pregnancies, never breast-feeding, and infertility are linked to increased incidence of ovarian cancer. Since these conditions may increase the number of ovulations in a woman's life-time, a unified hypothesis has been proposed to explain the interrelationships between OC and these contributory factors. It has been postulated that "incessant ovulation" leads to neoplastic transformation of HOSE cells. It is believed that following ovulation, ovarian epithelial cells undergo rapid proliferation to repair the ruptured epithelium. While the etiology of OC remains elusive, epidemiological observations have implicated ovarian steroids and/or gonadotropins, particularly when present at abnormal levels during and after menopause, as probable risk factors of OC. Understanding the role of hormones in ovarian carcinogenesis is of utmost importance to combat this deadly disease.

Project 4: Development of a highly sensitive and specific method for the early detection and a strategy for the early intervention of ovarian cancer

Ovarian carcinoma has the worst prognosis of any gynecological malignancy, due to the difficulty of early detection, the high metastatic potential of the tumor and the lack of highly effective treatment for metastatic disease. We have shown previously that lysophosphatidic acid (LPA) may represent a useful marker for the detection of ovarian cancer (5). The method used for LPA determination was a gas chromatographic method, which is cumbersome to perform. We have proposed to develop a mass spectrometry-based method to detect lysophospholipids in human body fluids (Task 1). This method will then be used to analyze lysolipids in blood samples collected from patients with ovarian cancer, other diseases, or healthy controls to determine whether one or more of these lipids may be useful for the detection of ovarian cancer (Task 1). In Task 2, we hypothesize that elevated levels of LPA in blood and ascites from patients with ovarian cancer are due to an abnormality of LPA production and/or degradation. We propose to study the enzymes controlling levels of LPA in ovarian cancer cells and/or body fluids from patients with ovarian cancer. If an abnormal enzymatic activity associated with ovarian cancer is identified, it may represent a target for early intervention, since LPA is likely to be involved in ovarian tumor cell growth, angiogenesis, and metastasis.

BODY

Project 1: Early genetic changes in human epithelial ovarian tumors

Task 1. Tissue collection, processing and microdissection (months 1-36): A total of 48 stage I epithelial ovarian carcinomas have been collected. Tissue collection will be continued in month 24-36.

Task 2. To perform loss of heterozygosity (LOH) studies on specific loci on chromosome 1p, 3p, 5q, 6q, 7q, 9p, 11p, 11q, 12p, 12q, 14q, 17p, 17q, 22q and Xq in microscopic stage I serous ovarian carcinomas by polymerase chain reaction (PCR) analysis of tandem repeat polymorphisms (months 1-36).

a). Tissue sectioning, and DNA extraction (months 1-12): Tissue sectioning, microdissection, and DNA extraction have been completed.

b). LOH study (months 3-36):

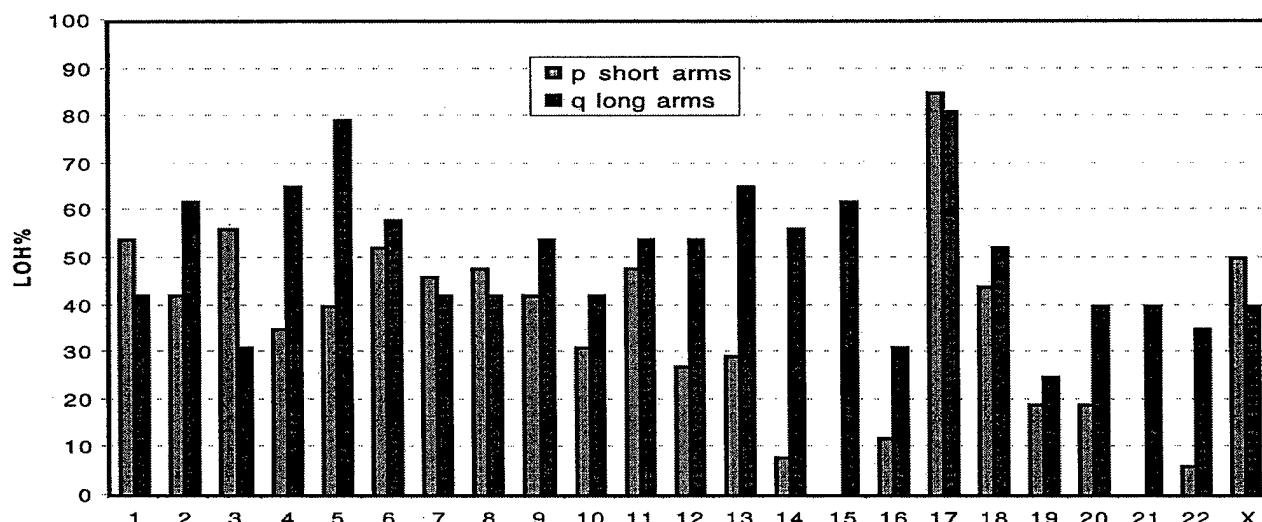


Figure 1. Frequency of loss of heterozygosity at each chromosome arm in 48 cases of stage I ovarian cancer

Using a high-throughput PCR-based method combined with laser capture microdissection and whole genome amplification techniques, we perform allelotyping on DNA isolated from 48 stage I sporadic epithelial ovarian cancer including 15 serous, 9 mucinous, 12 endometrioid, and 12 clear cell carcinomas. Among them, four are microscopically detected tumors. A total of 240 fluorescent-labeled microsatellite markers spanning the whole genome were used. The frequency of loss of heterozygosity (LOH) for each chromosome marker in all 48 cases was calculated (Figure 1). Chromosomal arms showed over 50% LOH rate include 1p, 2q, 3p, 4q, 5q, 6p and 6q, 9q, 11q, 12q, 13q, 14q, 15q, 17p and q, and 18q. Frequencies of LOH at specific loci were also determined and those over 30% were depicted in Table 1. These data indicate that high frequencies of LOH can be identified in multiple chromosomal arms in early stage ovarian cancer. Whether different subtypes of ovarian cancers have different allelic loss patterns is under investigation.

Table 1. Chromosomal regions with incidence (>30%) of LOH in stage I ovarian cancer

Chromosome arm	Markers	Regions	LOH/inf.	LOH%
1p	D1S199	1p36.13	12/36	33
2q	D2S2382	2q34-35	12/35	34
3p	D3S1304	3p25.3-25.2	13/37	35
3p	D3S1300	3p21.1	17/39	44
4q	D4S426	4q35.2	12/34	35
5q	D5S433	5q22	13/38	34
6p	D6S1574	6p24	18/43	42
6q	D6S287	6q21	13/34	38
6q	D6S441	6q24	14/32	44
6q	D6S264	6q25.2	19/32	59
8p	D8S550	8p23.1	10/32	31
8p	D8S258	8p22	12/36	33
9p	D9S288	9p24.1	12/38	31
9q	D9S158	9q34.3	9/27	33
11p	D11S4046	11p15	14/42	33
11p	D11S904	11p14.3	13/40	33
11p	D11S905	11p12	13/37	35
11q	D11S901	11q13.3-14.2	10/30	33
11q	D11S3120	11q24	10/26	38
12q	D12S351	12q21.32	12/34	35
12q	D12S1659	12q24.33	13/35	37
13p	D13S175	13p12-13q11	14/30	47
13q	D13S171	13q12.3	10/29	34
13q	D13S263	13q14.2	17/41	41
13q	D13S265	13q31.1	13/34	38
14q	D14S275	14q11.2	11/31	35
15q	D15S128	15q11.2-12	13/38	34
15q	D15S117	15q21.3	12/36	33
17p	D17S849	17p13.3	17/34	50
17p	D17S831	17p13.3	15/41	36
17p	D17S1828	17p13.1	19/45	42
17p	D17S1876	17p13.1	21/43	49
17p	D17S1791	17p12	14/41	34
17p	D17S799	17p12	19/38	50
17p	D17S921	17p12-q21.2	13/30	43
17p	D17S1857	17p12-q21.2	17/38	45
17p	D17S1824	17p12-q21.2	14/42	33
17q	D17S798	17q21.2	10/28	36
17q	D17S1795	17q21.3-22	13/40	32
17q	D17S787	17q21.3-22	17/38	45
17q	D17S944	17q22	12/27	44
17q	D17S1816	17q22-23	14/37	38
17q	D17S1862	17q24	21/45	47
17q	D17S836	17q25	11/26	42
17q	D17S784	17qter	11/32	34
20q	D20S196	20q13.2-13.31	12/38	31
21q	D21S266	21q22.3	12/33	36

Task 4. To identify differentially expressed genes in microdissected normal ovarian surface epithelial cells and Stage I ovarian carcinoma cells by RNA fingerprinting technique (months 12-36).

Perform RNA fingerprinting (months 12-24)

Characterize differentially expressed sequences (months 16-30)

1. Kim JH, Herlyn D, Wong KK, Park DC, Schorge JO, Lu KH, Skates SJ, Cramer DW, Berkowitz RS, Mok SC. Identification of Ep-CAM autoantibody in patients with ovarian cancer. *Clin Cancer Res.* 2003, in press.

Using the MICROMAX cDNA microarray system and RNA isolated from ovarian cancer cell lines and normal ovarian surface epithelial cells (HOSE), we identified a gene called the epithelial cell adhesion molecule (Ep-CAM) that exhibited a cancer-to-HOSE ratio of 444. Real time quantitative PCR analysis revealed significant overexpression of Ep-CAM mRNA in cancer cell lines ($P<0.001$) and microdissected cancer tissues ($p=0.035$), compared to that in cultured normal HOSE and microdissected germinal epithelium, respectively. Immuno-histochemical staining of paraffin block sections revealed that Ep-CAM expression was absent in stromal areas of normal ovaries or those with benign disease or cancer. In contrast, a gradient of expression was found in the germinal epithelium with ovaries from women with borderline or invasive cancer displaying the greatest level of expression, normal ovaries the least, and ovaries from women with benign tumors intermediate expression ($p<0.05$). No significant differences in Ep-CAM immuno-histochemical staining were observed among ovarian cancer samples with different histologic types and grades. Because Ep-CAM auto-antibody levels have been shown to be elevated in other cancers, such as colon, we examined levels of auto-antibody against Ep-CAM in patients with epithelial ovarian cancer and controls by enzyme-linked immunosorbent assay (ELISA). Ep-CAM auto-antibody levels (measured in units of absorbance at 450nm) were: 0.132 in 52 patients with ovarian cancer, 0.098 in 26 cases with benign gynecologic disease, and 0.090 in 26 normal women ($p<0.05$). When a cut-off value of 0.115 was used, the Ep-CAM auto-antibody assay showed a sensitivity of 71.2% and a specificity of 80.8% whereas the sensitivity and specificity of CA 125 measured in 52% of the same subjects were 84.6% and 88.5% with a CA 125 cut-off of 35U/ml. However, the Ep-CAM auto-antibody assay may be complementary to CA125, as indicated by low correlation coefficient and the fact that combining the test with CA 125 increased the sensitivity to 94.2% and specificity to 100.0%. This investigation has demonstrated the potential value of cDNA microarray analysis in identifying overexpressed genes in ovarian cancer, and suggests that the Ep-CAM auto-antibody may offer a biomarker for ovarian cancer with clinical usefulness.

Project 2: A Potential Serum Marker for Ovarian Cancer

Task 1: Investigation of expression of protease M in clinical samples:

1. Collection of samples: months 1 - 30

Samples are continuously collected by Dr. Samuel Mok and his associates.

2. Gene expression study: months 6 - 36

As stated in last annual report, we have generated protease M fusion protein and used it to immunize

mice. The first attempt of producing monoclonal antibodies by an outside company was not successful. We finally decided to produce monoclonal antibodies ourselves. After cell fusion and characterization of the clones, we identified five clones producing antibodies that are specific to protease M. One clone, 2D4, was chosen for immunohistochemical analysis of the archived samples (see the attached Manuscript). **Table 1** summarizes the protein expression data of protease M in ovarian tumor tissues. The overall F-test for the diagnostic groups was statistically significant ($P < 0.001$), indicating that mean immunostaining scores varies between diagnostic groups. The Fisher's pairwise comparison procedure was employed to compare the mean immunostaining scores at 5% level. The result shows that the healthy and benign tumors have significantly lower scores than the borderline and invasive tumors. Within the cancer (borderline and invasive) groups, there was no significant difference among histologic groups, as well as among different grades and stages. Representative figures for the immunohistochemical staining of normal ovary and different subtypes of ovarian tumor tissues are available in the manuscript.

Characteristics	No. of Patients	Mean of Scores	P Value
All patients	92	3.35	
Diagnostic category			
Healthy	3	0.00	
Benign	9	1.83	
Borderline	18	3.42	0.000
Invasive	62	3.71	
Histology of cancer			
Serous	44	3.44	
Mucinous	16	3.94	
Endometrioid	9	3.11	0.205
Clear Cell	4	5.00	
Mixed	7	4.14	
Tumor differentiation			
Borderline	18	3.42	
Grade 1	23	3.46	
Grade 2	7	4.07	0.792
Grade 3	29	3.64	
Stage			
I	24	3.48	
II	11	3.36	
III	31	3.61	0.965
IV	5	3.70	

Table 1. Expression of protease M in relation to histopathologic characteristics by immunohistochemical analysis.

In particular, we observed that in some mucinous tumor samples, there was strong hK6 staining in some apparently benign epithelia similar to the coexisting borderline tumors, or in borderline tumors coexisting with invasive tumors. As it is believed that mucinous ovarian carcinomas may arise from pre-existing benign or borderline lesions, it is very likely that elevated hK6 expression coincides with the early stages of ovarian cancer development.

Besides the evaluation of protein expression, we also determined kallikrein 6 mRNA levels between healthy human HOSE primary cell cultures and borderline and invasive tumors. For the borderline and invasive tumor samples other than serous subtype, tumor cells were microdissected from frozen sections by Laser Capture Microdissection (LCM) and total RNA was extracted from the captured tumor cells. The results in **Table 2** confirmed the significant protease M mRNA expression between normal HOSE primary cell cultures and tumor tissues ($P < 0.001$). For comparisons between different subtypes of

tumors, the F-test in the ANOVA was marginally significant ($P=0.054$), indicating that the mean expression levels were not the same for different subtypes of tumors. The result of the Fisher's pairwise comparison further indicated that mixed tumors had significantly lower mRNA expression levels than other types of tumors. The mean kallikrein 6 mRNA levels were not significantly different among various stages and grades of borderline and invasive tumor samples.

In conclusion, our immunohistochemistry and qRT-PCR data show that protease M is highly expressed in ovarian tumor tissues including early-stage and low-grade tumors, suggesting that elevated expression of protease M may be an early event during ovarian tumorigenesis.

Characteristics	No. of Patients	Mean of Expression	P Value
All patients	59	3.61	
Diagnostic category			
Healthy	7	-0.76	
Borderline	5	4.48	< 0.001
Invasive	46	4.05	
Histology of cancer			
Serous	35	4.61	
Mucinous	6	3.00	
Endometrioid	5	4.67	0.054
Clear Cell	2	4.42	
Mixed	4	1.64	
Tumor differentiation			
Borderline	4	4.78	
Grade 1	5	4.04	
Grade 2	9	3.62	0.819
Grade 3	33	4.26	
Stage			
I	10	4.21	
II	4	3.16	
III	28	4.26	0.757
IV	7	3.66	

Table 2. Human protease M mRNA expression in ovarian tissues.

Task 2: Substrate screening:

1. Enzymatic assays for protease M and other proteases: months 8 - 16

We have cloned the full-length protease M cDNA into the mammalian inducible expression vector *pcDNA6/TO/myc* (Invitrogen) in frame with the C-terminal hexahistidine (His) and *myc* tags and transfected into ovarian cancer cell line SKOV3, which has a deletion of the gene. By making use of the C-terminal His-tag, the recombinant protein was purified to near homogeneity from the low-serum conditioned medium by one step ProBond™ nickel affinity chromatographic column (Invitrogen) shown in **Figure 1A**. Before elution, the pro-enzyme was activated by mild trypsin treatment. The column was then extensively washed to remove the trypsin, and the bound proteins were eluted with elution buffers containing increasing concentrations of imidazole. Enzymatic activity of the purified protease M recombinant protein is demonstrated using both a commercial protease assay kit and a biotinylated fluorophosphonate (FP-biotin) assay. Measurable protease activity was detected with the recombinant protein purified from tetracycline-induced conditioned medium. A control sample prepared from uninduced conditioned medium and was similarly treated with trypsin did not show any protease activity (**Figure 1B**). Fluorophosphonate (FP) is a potent and specific inhibitor to serine hydrolases including

serine proteases (5). The reactivity of FP with serine hydrolases requires that the enzymes be in a catalytically active state. Hence, a FP linked to a small molecule reporter group (biotin) can serve as a potent and selective probe for monitoring simultaneously the activities of multiple serine proteases. As shown in **Figure 1C**, the FP-protease complexes resolved by SDS-PAGE and transferred to a PVDF membrane were revealed by avidin-horseradish peroxidase conjugate and chemiluminescence. Pre-treatments with heating abolished the interaction (**Figure 1C**, even lanes).

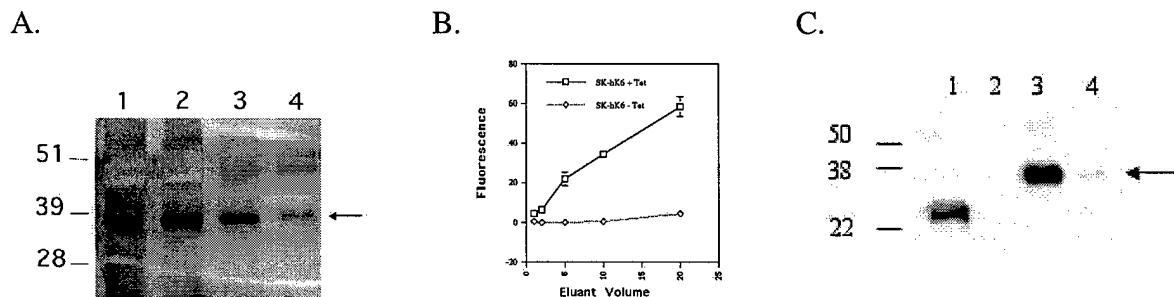


Fig. 1. Production of protease M recombinant protein.

- A. Silver-stained SDS-PAGE gel of bound proteins eluted with different concentrations of imidazole: lane 1: 50 mM; lane 2: 200 mM; lane 3: 350 mM; and lane 4: 500 mM. The recombinant proteins are marked by an arrow.
- B. Proteolytic assay using an EnzChek™ Protease Assay Kit (Molecular Probes). Proteolytic activity of the recombinant protease M protein eluted with 350 mM imidazole released the highly fluorescent BODIPY FL dye-labeled peptides. The plot shown is arbitrary fluorescence units versus eluant volumes (μl).
- C. FP-biotin activity assay. Lanes 1 and 2 are reactions with trypsin as controls. Lanes 3 and 4: reactions with active hK6. Lanes 2 and 4 were reactions with samples denatured by preheating to 80 °C. The hK6 band is indicated by an arrow.

2. Enzymatic assays in the presence of protease inhibitors: months 16 - 20

In progress.

3. Peptide library screening: months 14 - 24

We have given our protein samples to our collaborator, Dr. Ben Turk of Beth Israel Deaconess Medical Center. The first result was not satisfactory. We suspected that the protein amounts might not be enough for the assay. We are engineering a CHO serum-free system for robust synthesis and easy harvest of the recombinant protein. It is expected that enough purified protein will be produced using this system.

4. Confirmation of the optimal peptide motifs by enzymatic assays: months 25 - 30

Not started yet.

Task 3: Detection of protease M in patient blood:

1. Collection of samples and storage: months 1 - 24

Samples are continuously collected by Drs. Samuel Mok and Dan Cramer.

2. Development of detection methods: months 6 - 24

As mentioned in Task 1, we have prepared and purified protease M fusion protein and isolated five monoclonal antibody-producing clones. We have initiated collaboration with Dr. Dan Cramer in the OB/GYN Epidemiology Unit of our hospital. Dr. Cramer's laboratory belongs to the Early Detection Research Network (EDRN), a national scientific consortium. He will use our antibodies to screen pre-operative and post-operative serum samples from his serum bank that contains 190 cases, 297 benigns, 376 normals and samples from other gynecologic and non-gynecologic cancers. There will also be samples from Northwestern (248 samples), Southwestern and MD Anderson. Dr. Cramer is also carrying out a study to collect prospective samples from 1700 high-risk subjects, which can also be available. Screening will be performed by Dr. Patrick Sluss at the Clinical Core of Massachusetts General Hospital, who has extensive experience in developing immunoassays. The first assay to be developed will be a competitive labeled antigen immunoassay, which depends on the competition between a labeled antigen and its unlabeled counterpart in the serum for binding to a limited amount of specific antibody. We are also characterizing the other monoclonal antibodies so that Dr. Sluss can develop a more sensitive and specific sandwich-based immunoassay. Dr. Steven Skates of the Biostatistics Core will analyze the serum hK6 data together with other parameters such as CA125 levels, age, pre- or post-menopause, chemoresponse, and survival data. Serum hK6 values will be evaluated for any predictive value by itself or in conjunction with other biomarkers like CA125. With the participation of Dr. Sluss and the expanded scope of the multi-marker screening, we are very excited that evaluation of multi-markers including protease M should improve the diagnostic prospect of ovarian cancer.

3. Assays on the blood samples: months 25 - 30

Will be started very soon.

4. Data analysis: months 31 - 36

Not started yet.

Project 3: Hormones as etiological factors of ovarian carcinogenesis

The first objective is to determine the efficacies of selected estrogens, to achieve this the HOSE cells will be treated with increasing concentrations of estrogen for five days. The cell proliferation will be measured by MTT assay. To study the synergistic effect of FSH and estrogens cells will be cultured in the absence or presence of FSH and HOSE cell proliferation will be studied. To ascertain whether their mitogenicities are mediated via estrogen receptors receptor blocker will be used.

The second objective is to determine whether the 3 selected estrogens have direct oncogenic potentials and if they could be enhanced by FSH and blocked by the antiestrogen, ICI 182, 780. The HOSE cells will be plated and exposed to different doses of estrogen for two weeks. Soft agar assay will be used to study the transformation potential of estrogens. In a parallel experiment FSH will be added along with estrogen to study the synergistic effect on cell transformation.

The third objective is to pick up a hormonal milieu that will produce the highest frequency of *in vitro* transformation. To ascertain whether progesterone and DHEA exert anti-tumorigenic action by blocking the estrogen and/or FSH-induced neoplastic transformation of HOSE cells, *in vitro* transformation assay will be used to assay the ability of progesterone and DHEA in inhibiting the estrogen-gonadotropin-induced transformation of HOSE cells.

Results:

Estradiol stimulated cell proliferation is inhibited by antiestrogen: When increasing concentrations (10^{-11} - 10^{-6} M) of estrone (E1) or estradiol (E2) were added to primary HOSE 639, HOSE 770, HOSE 783, HOSE 785, and immortalized normal HOSE 642, HOSE 301, HOSE 306, HOSE 12-12, in culture, a dose dependent rise in cell proliferation was observed. About ten to fourteen fold increase was noted by 10^{-6} M E1 or E2 in HOSE 639, HOSE 770, HOSE 783, HOSE 785 cell lines compared to six fold increase in normal immortalized lines HOSE 642, HOSE 301, HOSE 306 and HOSE 12-12 cell lines. E1 and E2 were equally effective in causing cell proliferation in all cell lines except HOSE 12-12 cell line where E1 showed a significant enhancement of cell proliferation compared to E2. Cell lines cultured with FSH and estradiol showed significant cell growth but no additive effect was seen in any cell line tested. The ICI considered as pure antiestrogen, functions specifically by binding to and inactivating the estrogen receptor. When primary and immortalized HOSE cells were incubated with 10^{-8} M E2 A marked enhancement of cell proliferation was seen in all the cell lines with E2 and when cells were cultured with E2 and two doses (10^{-5} and 10^{-4} M) of ICI for 5 days, addition of ICI to cultures along with E2 markedly attenuated cell proliferation.

FSH and estrogen combination enhance colony formation of HOSE cells. - The first criterion used to select transformed HOSE cells following exposure to estrogen is their ability to proliferate on soft agar. We have established a standard protocol to test the carcinogenicity of hormones on HOSE cells. Briefly, HOSE cells were plated at low density in 24-well plates and exposed to different doses of either estrogen (DES), FSH or combination of both FSH and DES for 14 days. After treatments, cells were removed from the 24-well plates with trypsin and replated in 6-well plates for expansion of potential transformants. Once the cell cultures reached confluence in 6-well plates they are removed and subjected to soft-agar growth selection (Freshney, 1994).. After two-four weeks of soft agar selection, individual clones proliferating on soft agar plates were removed, expanded, and stored for further investigations.

Using this protocol, we discovered that HOSE cells, exposed continuously to diethylstilbestrol (DES), a potent synthetic estrogen, at concentrations between 10^{-9} to 10^{-8} M for 14 days, had acquired ability to grow on soft agar (Table 1).

Approximately 4-6, 3-5 and 8-10 colonies were found in a total of 10^4 FSH treated, DES treated and FSH+DES treated cells plated on soft agar respectively. Untreated HOSE cells did not form any soft-agar colonies suggesting little or no spontaneous transformation activity. At present, the FSH+DES-induced colonies derived from these preliminary experiments are under passaging to establish stable lines.

Hormones	FSH	DES	FSH+DES
# of colonies	4-6	3-5	8-10

Table 3. Number of colonies obtained by treatment of HOSE Cells with different hormones.

Project 4: Development of a highly sensitive and specific method for the early detection and a strategy for the early intervention of ovarian cancer

Task 1. Develop a highly sensitive and specific method for the early detection of ovarian cancer, Months 1-36

Task 2. Develop a strategy for the early intervention of ovarian cancer, Months 1-36

Work accomplished as proposed in Task 1 in the last year

We have completed lipid analyses in 23 patients with ovarian cancer, 9 patients with breast cancer, and 23 healthy controls.

Summary Statistics

Table 1 summarizes the various lipid markers assayed and compares levels obtained from ovarian cancer patients to levels in patients with breast cancer and healthy subjects using the Wilcoxon rank sum test. The markers that are statistically significantly different between groups at the $p<.01$ level are flagged in the last column. As can be seen from this table ovarian cancer patients and non-ovarian cancer patients differ with respect to all markers except S1P, LPAF, the saturated species of LPC (LPC 16:0 and LPC 18:0), and most of the LPC-based ratios.

Correlations

Table 2 summarizes qualitatively the correlations between the various species totals. Combining all patients, alkenyl LPA, alkyl LPA, and LPA are all positively correlated with each other, as are LPI and LPA and LPC and LPAF (Pearson correlations).

Discrimination

Considering the markers listed in Table 1 separately, Table 3 summarizes the sensitivity and specificity at empirically defined “optimum” cut points (i.e. the cut points that maximize sensitivity and specificity) for those markers found to be statistically significantly different between the groups at $p<.01$. Cut points that result in $>90\%$ sensitivity and specificity, respectively, are highlighted in a bold, italic font. For ovarian cancer “regular” LPA and total LPA (all sources) appear to have good discriminatory power. For example, using a cutoff of 1.0 for total “regular” LPA results in 91% sensitivity (2 ovarian cancers are misclassified) and 100% specificity (no breast cancer patient or healthy control had a “regular” LPA >1.0). Similar results are obtained with respect to the saturated portion of “regular” LPA using a cutoff of 0.77. It should be noted, however, that sensitivity and specificity are highly dependent on the cutoff chosen. For example, changing the cutoff for saturated LPA from 0.77 to 0.75 reduces the specificity from 100% to 94%. Using multivariable logistic regression models and discriminate analysis these markers can be combined in various ways to potentially improve upon the sensitivity and specificity. Figures 1 summarizes these results graphically.

Table 1. Summary Statistics

Marker	Ovarian Cancer (n=23)			Breast Cancer (n=9)			Healthy Controls (n=23)			
	Mean ± s.d.	Median	Range	Mean ± s.d.	Median	Range	Mean ± s.d.	Median	Range	p ¹
S1P	0.15 ± 0.15	0.11	0-0.69	0.13 ± 0.15	0.02	0-0.38	0.06 ± 0.04	0.05	0-0.13	
16:0 Alkenyl LPA	0.03 ± 0.04	0.02	0-0.16	0.01 ± 0.01	0.01	0.003-0.02	0.02 ± 0.03	0.01	0-0.11	
18:0 Alkenyl LPA	0.07 ± 0.07	0.04	0.002-0.26	0.01 ± 0.01	0.01	0-0.04	0.03 ± 0.05	0.01	0.001-0.19	
Total Alkenyl LPA	0.10 ± 0.10	0.06	0.003-0.42	0.02 ± 0.01	0.02	0.01-0.05	0.04 ± 0.07	0.02	0.01-0.29	
16:0 Alkyl LPA	0.10 ± 0.10	0.09	0-0.42	0.03 ± 0.03	0.01	0.003-0.07	0.04 ± 0.08	0.02	0-0.32	
18:0 Alkyl LPA	0.06 ± 0.05	0.06	0-0.19	0.01 ± 0.01	0.01	0.001-0.04	0.02 ± 0.03	0.01	0-0.11	
Total Alkyl LPA	0.16 ± 0.15	0.18	0-0.62	0.04 ± 0.04	0.02	0.01-0.10	0.06 ± 0.10	0.03	0.004-0.42	
Alkyl+Alkenyl LPA	0.26 ± 0.24	0.29	0.01-1.04	0.07 ± 0.05	0.03	0.002-0.14	0.10 ± 0.18	0.05	0.02-0.72	
16:0 LPA	1.57 ± 1.40	1.06	0.47-6.60	0.11 ± 0.09	0.07	0.01-0.26	0.17 ± 0.20	0.08	0-0.61	
18:0 LPA	0.61 ± 0.50	0.41	0.08-1.93	0.04 ± 0.03	0.02	0.005-0.10	0.07 ± 0.07	0.04	0-0.23	
Total Saturated	2.17 ± 1.86	1.76	0.56-8.53	0.14 ± 0.12	0.08	0.02-0.32	0.24 ± 0.26	0.10	0-0.77	
18:1 LPA	0.39 ± 0.39	0.30	0-1.77	0.03 ± 0.03	0.02	0.004-0.08	0.03 ± 0.02	0.02	0-0.08	
18:2 LPA	0.31 ± 0.32	0.17	0.01-1.10	0.04 ± 0.03	0.04	0.004-0.09	0.08 ± 0.07	0.04	0-0.25	
20:4 LPA	0.22 ± 0.19	0.13	0.01-0.68	0.02 ± 0.02	0.02	0.001-0.05	0.05 ± 0.05	0.05	0-0.17	
22:6 LPA	0.14 ± 0.14	0.09	0.006-0.42	0.02 ± 0.01	0.01	0.001-0.04	0.01 ± 0.01	0.01	0-0.03	
Total Unsaturated	1.05 ± 0.89	0.64	0.13-3.86	0.11 ± 0.09	0.10	0.02-0.26	0.17 ± 0.09	0.17	0-0.40	
Total "Reg." LPA	3.23 ± 2.52	2.95	0.69-12.38	0.25 ± 0.21	0.18	0.03-0.57	0.42 ± 0.28	0.30	0.06-0.95	
Unsat.:Sat. Ratio³	0.37 ± 0.21	0.45	0.10-0.92	0.80 ± 0.25	0.71	0.50-1.22	1.84 ± 1.97	1.41	0.23-6.96	
LPA - All Sources	3.49 ± 2.53	3.10	0.80-12.50	0.32 ± 0.26	0.20	0.06-0.70	0.52 ± 0.43	0.32	0.11-1.65	
Unsat.:Sat. Ratio	0.47 ± 0.28	0.32	0.08-1.06	0.52 ± 0.17	0.49	0.34-0.94	1.57 ± 2.28	0.90	0-10.30	
16:0 LPI	0.57 ± 0.69	0.28	0-2.84	0.05 ± 0.03	0.04	0.03-0.13	0.10 ± 0.04	0.09	0.03-0.19	
18:0 LPI	2.09 ± 3.63	0.73	0-14.58	0.15 ± 0.09	0.12	0.04-0.33	0.37 ± 0.14	0.39	0.13-0.66	
Total Saturated	2.66 ± 4.27	1.02	0-17.41	0.20 ± 0.12	0.16	0.08-0.46	0.47 ± 0.18	0.49	0.16-0.85	

Marker	Ovarian Cancer (n=23)			Breast Cancer (n=9)			Healthy Controls (n=23)		
	Mean \pm s.d.	Median	Range	Mean \pm s.d.	Median	Range	Mean \pm s.d.	Median	Range
20:4 LPI	0.28 \pm 0.28	0.19	0-0.89	0.10 \pm 0.09	0.07	0.004-0.31	0.18 \pm 0.14	0.20	0.01-0.49
Total LPI	2.94 \pm 4.34	1.47	0-17.74	0.31 \pm 0.21	0.24	0.08-0.77	0.65 \pm 0.27	0.65	0.17-1.34
16:0 LPC	24.6 \pm 15.3	20.7	5.9-64.5	44.3 \pm 7.3	45.5	29.8-53.3	28.6 \pm 16.1	28.7	10.2-63.8
18:0 LPC	8.9 \pm 6.4	6.4	2.0-24.7	12.8 \pm 2.1	12.2	8.7-15.3	9.1 \pm 4.4	7.7	0.54-16.2
Total Saturated	34.1 \pm 22.1	28.4	8.1-93.2	57.5 \pm 8.4	58.3	38.7-65.8	38.1 \pm 20.1	36.5	13.3-74.9
18:1 LPC	5.7 \pm 5.6	4.5	0-23.2	15.0 \pm 3.4	15.5	7.9-20.5	10.9 \pm 8.6	9.6	0.2-27.7
18:2 LPC	4.2 \pm 3.1	2.9	0.1-10.8	21.8 \pm 5.3	22.5	12.5-30.8	18.4 \pm 15.4	16.6	0.1-43.4
20:4 LPC	2.8 \pm 3.3	1.1	0-9.9	15.3 \pm 3.6	15.0	9.6-20.7	9.8 \pm 9.5	9.9	0.1-36.1
Total Unsaturated	15.0 \pm 11.2	13.5	1.4-38.7	61.6 \pm 12.7	58.1	40.2-87.2	44.0 \pm 35.8	49.6	0.8-108.6
Total LPC	49.1 \pm 29.4	36.7	11.1-127.6	119.0 \pm 18.8	123.0	78.8-150.2	82.1 \pm 54.3	91.1	16.0-166.5
Unsat:Sat. Ratio	0.51 \pm 0.44	0.38	0.06-1.98	1.07 \pm 0.18	1.04	0.88-1.38	1.00 \pm 0.68	1.12	0.05-2.41
16:0 to 18:2 Ratio	17.57 \pm 37.64	5.89	1.50-181.29	2.10 \pm 0.39	2.19	1.41-2.66	16.54 \pm 43.84	1.78	0.77-155.44
16:0 to 18:1 Ratio ⁴	8.83 \pm 15.86	3.74	1.73-71.59	3.04 \pm 0.57	3.01	2.33-3.94	9.30 \pm 19.90	2.98	1.69-79.16
16:0 to 18:0 Ratio	2.98 \pm 0.81	2.77	1.75-4.61	3.51 \pm 0.64	3.38	2.54-4.47	4.12 \pm 4.58	2.88	1.70-23.54
16:0 to 20:4 Ratio ⁵	38.11 \pm 57.25	13.09	1.26-208.81	3.04 \pm 0.87	2.58	2.12-4.74	17.23 \pm 23.75	3.82	1.21-81.32
16:0 to 22:6 Ratio	18.15 \pm 15.03	11.59	2.37-62.15	5.24 \pm 1.85	4.73	2.45-7.85	15.85 \pm 15.16	7.72	2.25-58.47
18:0 to 18:2 Ratio	6.43 \pm 13.33	2.24	0.35-63.59	0.61 \pm 0.13	0.55	0.47-0.90	8.18 \pm 22.82	0.50	0.03-91.02
L-PAF	0.14 \pm 0.13	0.10	0.02-0.54	0.31 \pm 0.10	0.27	0.18-0.49	0.14 \pm 0.08	0.13	0.03-0.29

¹ Difference between breast cancer patients and healthy controls is statistically significant at p<.01 (Wilcoxon rank sum test)

² Difference between ovarian cancer patients and healthy controls/breast cancers is statistically significant at p<.01 (Wilcoxon rank sum test)

³ n=21 control patients with “Regular” saturated LPA>0

⁴ n=19 ovarian cancer patients with LPC 18:1>0

⁵ n=22 ovarian cancer patients with LPC 20:4>0

⁶ Healthy controls versus breast cancer patients

⁷ Ovarian cancer patients versus controls plus breast cancer patients

Table 2. All Patients (n=55)

	LPC	LPAF	LPI	Total LPA ²	Reg. LPA	Alkenyl LPA	Alkyl LPA	S1P
S1P	no	no	Yes (+)	no	no	no	no	
Alkyl LPA	Yes (-)	<i>p=.01</i> (-)	no	Yes (+)	no	Yes (+)		
Alkenyl LPA	Yes (-)	no	no	Yes (+)	no			
Reg. LPA	<i>p=.01</i> (-)	no	Yes (+)	Yes (+)				
Total LPA ²	Yes (-)	no	Yes (+)					
LPI	no	no						
LPAF	Yes (+)							
16:0 to 18:0	no	no	no	no	no	no	no	
16:0 to 18:1 ³	Yes (-)	no	no	no	Yes (+)	Yes (+)	no	
16:0 to 18:2	<i>p=.01</i> (-)	no	no	no	Yes (+)	Yes (+)	no	
16:0 to 20:4 ⁴	no	no	no	no	no	no	no	
16:0 to 22:6	Yes (-)	Yes (-)	no	no	Yes (+)	Yes (+)	no	
18:0 to 18:2	<i>p=.01</i> (-)	no	no	no	Yes (+)	Yes (+)	no	

¹ “Yes” indicates the Pearson correlation is statistically significantly different from 0 at p<.01

² LPA from all sources – alkyl+alkenyl+“regular” LPA

“+” indicates a positive correlation and “-“ indicates an inverse relationship

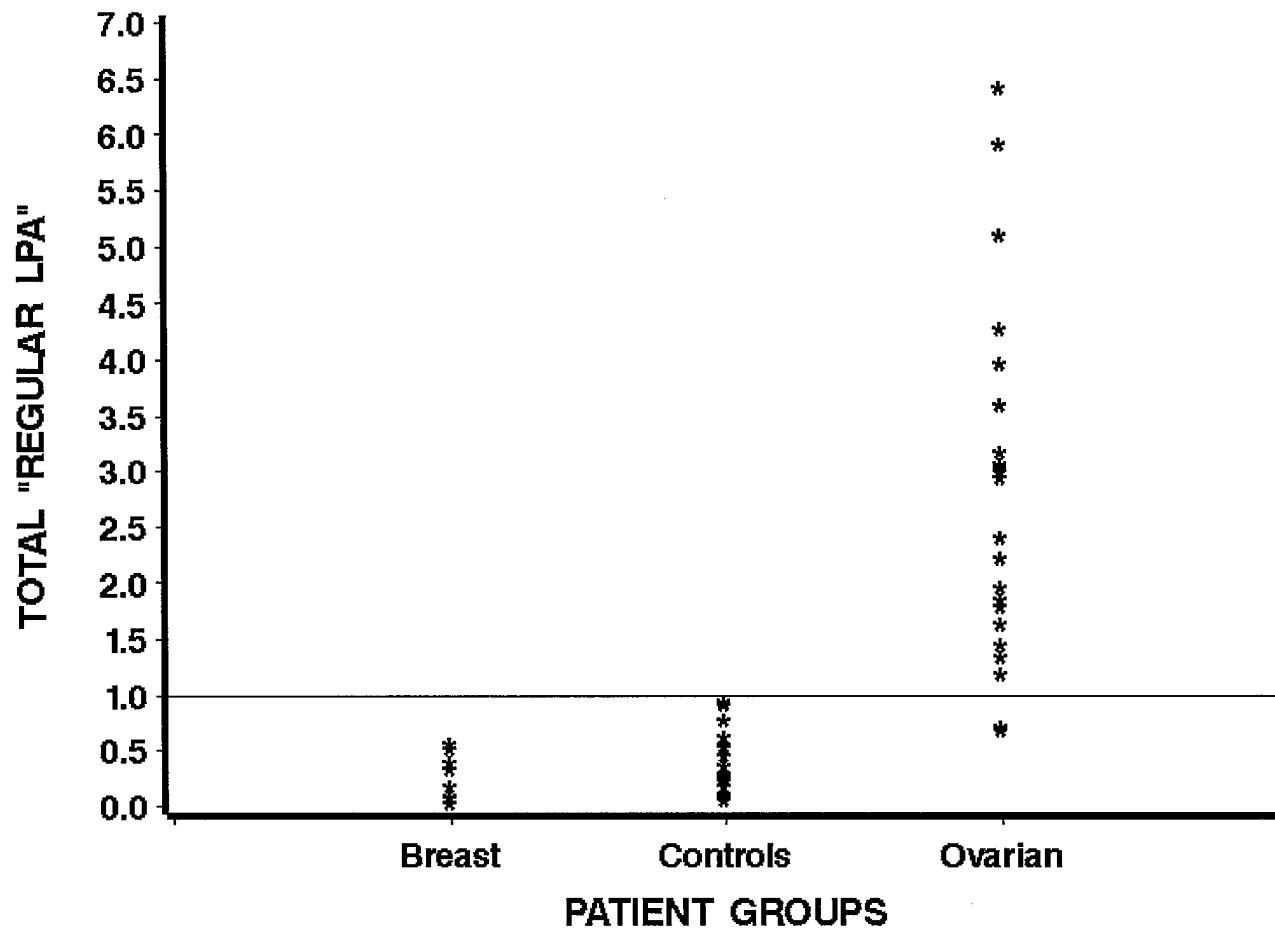
³ n=51 subjects with LPC 18:1>0

⁴ n=54 subjects with LPC 20:4>0

Table 3. Cutpoints for Classifying Ovarian Cancer

	Cut Point	Sensitivity	Specificity
18:0 Alkenyl LPA	>0.03	61%	84%
Total Alkenyl LPA	>0.035	65%	84%
16:0 Alkyl LPA	>0.04	65%	81%
Total Alkyl LPA	>0.06	65%	81%
Alkyl+Alkenyl LPA	>0.10	70%	81%
16:0 LPA	>0.62	91%	97%
18:0 LPA	>0.20	91%	97%
Total Saturated	>0.77	91%	100%
18:1 LPA	>0.10	78%	100%
18:2 LPA	>0.16	52%	94%
20:4 LPA	>0.11	70%	91%
22:6 LPA	>0.05	65%	100%
Total Unsaturated	>0.40	83%	97%
Total "Reg." LPA	>1.0	91%	100%
Unsat.:Sat. Ratio³	<0.71	35%	81%
LPA – All Sources	>1.6	91%	97%
16:0 LPI	>0.11	78%	91%
18:0 LPI	>0.50	78%	91%
Total Saturated	>0.65	78%	94%
Total LPI	>1.0	70%	97%
18:1 LPC	<6.5	65%	72%
18:2 LPC	<12.0	100%	72%
20:4 LPC	<5.0	78%	72%
Total Unsaturated	<30.0	87%	72%
Total LPC	<75.0	78%	66%
Unsat.:Sat. Ratio	<0.85	87%	72%
16:0 to 18:2 Ratio	>2.7	87%	75%
16:0 to 20:4 Ratio	>4.0	77%	66%
18:0 to 18:2 Ratio	>1.37	70%	75%

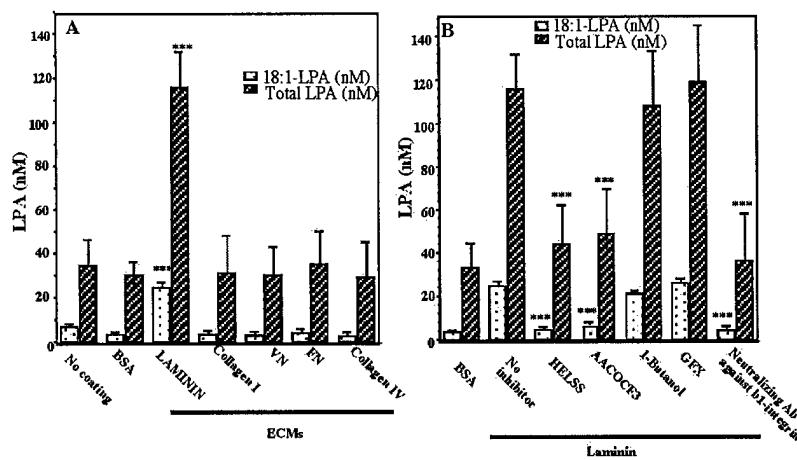
Figure 1. Total "Regular" LPA



Work accomplished as proposed in Task 2 in the last year

1. The first mammalian lysophospholipase D (lyso-PLD) has been identified recently by two Japanese groups (5,6). We have found that a lysoPLD-like activity is present in ascites from patients with ovarian cancer (Ref 4; appendices).

2. We have found that when HEY cells were incubated in 6-well plates coated with BSA (as a control) or different extracellular matrix (ECM)s, laminin, *but not any other ECMs*, stimulated an approximately 4-fold increase of LPA (Fig. 2A). To study the mechanisms of LPA produced by laminin, we tested a number of phospholipase inhibitors: HELSS (a iPLA₂ specific inhibitor, AACOCF₃ (an iPLA₂ and cPLA₂ inhibitor), 1-butanol (a PLD inhibitor), and GF 109203X (GFX; a PKC inhibitor). Both HELSS and AACOCF₃ but not 1-butanol and GFX, significantly inhibited the LPA production (Fig. 2B). These results suggest that an iPLA₂, but not PLD, or a GFX-sensitive PKC, is involved in the laminin-induced LPA production. Importantly, we found that the β_1 neutralizing antibody completely blocked the production of LPA (Fig. 2B), indicating that laminin-induced LPA production via activating a β_1 integrin(s).



KEY RESEARCH ACCOMPLISHMENTS

- We demonstrated that early stage ovarian cancers, including microscopic diseases, have allelic losses at multiple chromosomal sites.
- We identified Ep-CAM autoantibody as a potential serum biomarker for ovarian cancer.
- We have confirmed that LPA is a highly sensitive marker for detection of ovarian cancer.
- Gene expression analysis of the tumor samples has demonstrated that protease M is highly expressed in ovarian tumors of various stages and subtypes but not in the normal ovarian epithelial cells.
- Protease M recombinant protein has been produced and preliminary studies have shown that it exhibits protease activity. The activity to peptide library will be determined.
- Monoclonal antibodies specific to protease M have been established. Serum screening protocol will be developed very soon.
- In addition, we have found that alkenyl-, acyl-LPAs, and lysophosphatidylinositol (LPI), may further increase the specificity and sensitivity of the LPA test. A US patent (#6,451,609) covering these findings has been issued.
- We have identified that iPLA₂ and lysoPLD may be important for LPA production from ovarian cancer cells. Further studies will reveal whether they are important target for the early prevention or the treatment of ovarian cancer.
- We have shown here for the first time that an ECM molecule is able to stimulate LPA production. This is also the first work to show that HEY ovarian cancer cells are able to produce LPA when stimulated by a physiological stimulus. This LPA production is potentially related to tumor cell metastasis. We believe that these findings are highly novel and significant to ovarian cancer.

REPORTABLE OUTCOMES

Project 1: Early genetic changes in human epithelial ovarian tumors

Manuscript:

Kim JH, Herlyn D, Wong KK, Park DC, Schorge JO, Lu KH, Skates SJ, Cramer DW, Berkowitz RS, Mok SC. Identification of Ep-CAM autoantibody in patients with ovarian cancer. Clin Cancer Res. 2003, in press.

Funding applied for based on the work supported by this award:

"Prognostic markers for ovarian cancer" (7/1/03 -6/30/07)

Principal Investigator: Samuel C. Mok

Agent : NIH

Type: R33

To identify prognostic markers for epithelial ovarian cancers.

"Genetic changes in early stage ovarian cancer" in "Ovarian Cancer SPORE" (12/1/03 -11/30/08)

Principal Investigator: Samuel C. Mok

Agent : NIH

Type: P50

To identify markers for early detection of epithelial ovarian cancers.

Project 2: A Potential Serum Marker for Ovarian Cancer

We have generated monoclonal antibodies specific to protease M and ovarian cell lines that are inducible to produce protease M recombinant protein. A manuscript entitled "Characterization of human kallikrein 6/protease M expression in ovarian cancer" has been submitted to Clinical Cancer Research and appended herein. We have also based on the data resulted from this support applied for funding to continue this project.

Project 3: Hormones as etiological factors of ovarian carcinogenesis

Manuscript:

1. Syed V, Ulinski G, Mok SC, Yiu GK, Ho SM. Expression of gonadotropin receptor and growth responses to key reproductive hormones in normal and malignant human ovarian surface epithelial cells. *Cancer Res.* 2001 Sep 15;61(18):6768-76.
2. Syed V, Ulinski G, Mok SC, Ho SM. Reproductive hormone-induced, STAT3-mediated interleukin 6 action in normal and malignant human ovarian surface epithelial cells. *J Natl Cancer Inst.* 2002 Apr 17;94(8):617-29.
3. Ho SM, Lau KM, Mok SC, Syed V. Profiling follicle stimulating hormone-induced gene expression changes in normal and malignant human ovarian surface epithelial cells. *Oncogene.* 2003 Jul 3;22(27):4243-56.
4. Syed V, Ho SM. Progesterone-induced apoptosis in immortalized normal and malignant human ovarian surface epithelial cells involves enhanced expression of FasL. *Oncogene (in press)*

Project 4: Development of a highly sensitive and specific method for the early detection and a strategy for the early intervention of ovarian cancer

Peer-reviewed publications

1. Baudhuin LM, Kristina KL, Lu, J, and Xu Y. Activation induced by LPA and S1P requires both MEK and p38 MAP kinase and is cell-line specific. *Mol Pharmacol* 62, 660-671, 2002.
2. Lu J, Xiao Y, Baudhuin LM, Hong G, and Xu, Y. Role and Signaling Pathways of Ether-linked Lysophosphatidic Acids in Ovarian Cancer Cells. *J. Lipid Res.* 43, 463-476, 2002.
3. Zhu, K., Baudhuin, L., Hong, G. Williams, F.S., Cristina, K.L., Kabarowski, J.H.S., Witte, O.N. and Xu, Y Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein coupled receptor, GRP4. *J Biol Chem.* 276(44):41325-41335, 2001.

Review papers

1. **Xu Y.** Sphingosylphosphorylcholine and lysophosphatidylcholine: G protein coupled receptors and receptor-mediated signal transduction. *Biochem Biophys Acta*, 1582, 81-88, 2002.
2. **Xu Y**, Xiao Y, Zhu K, Baudhuin LM, Lu J, Hong G, Kim K-s, Cristina KL, Song L, Williams FS, Elson P, and Belinson J. Unfolding the pathophysiological role of bioactive lysophospholipids. Current drug targets-immune, endocrine & metabolic disorders. In press
3. Bauhuin L, Xiao Y, and **Xu Y.** SPC/LPC receptors Handbook of Cellular Signaling. In press.

Abstracts:

1. Saubhik Sengupta, Yi-Jin Xiao, and Yan Xu Production of Lysophosphatidic Acid (LPA) by Laminin – Integrin Interaction is the key for Laminin induced Ovarian Cancer cell Migration. 22nd Annual Research Day at the Cleveland Clinic Foundation. Oct. 17, 2002.
2. Guiying Hong and Yan Xu OGR1 overexpression modulates cell-cell and cell-matrix interactions and induce cell apoptosis. 22nd Annual Research Day at the Cleveland Clinic Foundation. Oct. 17, 2002
3. Yi-jin Xiao, Benjamin Schwartz, Donna Fife, April While, Alexander Kennedy, Jerome Belinson, and Yan Xu. Analyses of LPA in peritoneal washings enhances of the sensitivity and specificity of ovarian cancer early detection. 22nd Annual Research Day at the Cleveland Clinic Foundation. Oct. 17, 2002
4. Baudhuin LM and Xu Y. Activation of Akt by LPA and S1P is Dependent on the Activities of both ERK and p38 MAP kinases in HEY Ovarian Cancer Cells. 18th UICC International Cancer Congress (6/30-7/7/02 Oslo, Norway). Int. J. Cancer Supplmnt 13, P 806, 2002
5. **Xu Y** and Baudhuin LM. Sphingosine-1-phosphate-mediated activation of Akt through Edg-3, but not Edg-1, requires the activation of platelet-derived growth factor receptor. AACR 93rd Annual meeting, p1011 (April 6-10, 2002, San Francisco, CA)
6. **Xu Y**, Lu J, Xiao Y, Baudhuin LM, and Hong G. Role and Signaling Pathways of Ether-linked Lysophosphatidic Acids in Ovarian Cancer Cells. AACR 93rd Annual meeting, p255 (April 6-10, 2002, San Francisco, CA)

Presentations:

1. "Role and signaling pathways of lysophospholipids in cancer" (an invited talk at Mayo Clinic) (9/24/02)
2. "Biomarker detection in breast cancer" Breast cancer conference, The Taussig Cancer Center, 9/19/02.
3. "Bioactive lysophospholipids, their receptors, and cancers" The Netherlands Cancer Institute, Amsterdam, Netherlands, (7/8/02).
4. "Receptors for SPC and LPC" (an invited talk at the Annual Meeting GERLI 2000) (4/18/01) Nantes, France.
5. "Role of signaling pathways of lysophospholipids in cancer" (an invited talk in the Department of Pathology, Case Western Research University) (4/29/02).
6. "Role of signaling pathways of lysophospholipids in cancer" (ICOS LLC; Alameda, CA; 2/25/02).
7. "Role of signaling pathways of lysophospholipids in cancer" (Department of Pharmacology, Case Western Reserve University; 2/21/02).
8. "The potential pathological roles of LPA in ovarian cancer" (an invited talk at the 74th Annual

Meeting of the Japanese Biochemical Society; 10/27/01).

- 9. "G protein coupled receptors for LPC and SPC" (a talk at the CCF Retreat; Maumee Bay, OH; 9/11/01).
- 10. "G protein coupled receptors for LPC and SPC" (Beijing University, Beijing, P.R. China; 9/25/01).
- 11. "Role of signaling pathways of lysophospholipids in cancer" (Institute of Animal, The Chinese Academy of Science; Beijing, P.R.China; 9/28/01).
- 12. "Role of signaling pathways of lysophospholipids in cancer" (Beijing Normal University, P.R.China; 10/5/01).

Patent

Xu Y, Xiao Y US patent 6,451,609 9/17/2002; Method of Detecting Gynecological Carcinomas

Degree obtained that is supported in part by this award

Dr. Linnea Baudhuin has obtained her Ph.D. in May 2002

Funding applied for based on the work supported by this award:

"Novel lipid signaling pathways in ovarian cancer cells"

Principal Investigator: Yan Xu

Agent: To be submitted as a revision to NIH by 11/01/02

Type: RO1

Understand the signaling mechanisms of lipid molecules in ovarian cancer cells and identify novel targets to be used as potential therapeutics for the treatment of cancer.

CONCLUSIONS

Project 1: Early genetic changes in human epithelial ovarian tumors

We showed that high frequencies of LOH can be identified in multiple chromosomal arms in early stage ovarian cancer. Whether different subtypes of ovarian cancers have different allelic loss patterns is under investigation. We also demonstrated that Ep-CAM autoantibody is a candidate biomarker for ovarian cancer, which may have clinical usefulness.

Project 2: A Potential Serum Marker for Ovarian Cancer

In spite of several unexpected incidences and shortcomings, we were able to accomplish some of the tasks listed in the application. Analysis of the tumor samples has demonstrated that protease M is highly expressed in ovarian tumors of various stages and subtypes but not in the normal ovarian epithelial cells. The findings that elevated protease M expression in early-stage and low-grade tumors and in apparently benign epithelium coexisting with borderline and invasive tumors suggest that elevated protease M expression occurs early in tumorigenesis and tumor progression. **As monoclonal antibodies can be continuously produced without change of quality, the immunoassay protocol developed by us will be suited for future standard large-scale screenings.** This is important for the development of screening tools for early detection of ovarian cancer. Characterization of protease M recombinant protein suggested that it exhibits protease activity. Pending on funding, further delineation of the enzymatic properties of protease M will facilitate understanding of this novel protease and

development of useful reagents for early detection and intervention of ovarian cancer.

Project 3: Hormones as etiological factors of ovarian carcinogenesis

The first objective was to determine the efficacies of estrogens and to study the synergistic effect of FSH and estrogen on cell proliferation. This aim is successfully completed and extended by looking at the effect of estrogen on cell proliferation in primary cell lines in addition to immortalized cells. Our results demonstrated that all the cell lines responded equally well to E2 and E1 except HOSE 12-12-cell line, where E1 was more effective than E2 in inducing cell proliferation. Of particular interest is that primary cells are more responsive to E2 and E1 than immortalized cells. Furthermore, No synergism was observed when cultures were challenged simultaneously with FSH and E2. The second aim has been successfully completed. We have demonstrated that combination of FSH and DES resulted in greater number of colonies.

Project 4: Development of a highly sensitive and specific method for the early detection and a strategy for the early intervention of ovarian cancer

We have made important accomplishment in developing a method of detecting and a strategy for the early intervention of ovarian cancer. The newly developed ESI-MS-based method is highly sensitive, reproducible and quantitative. We confirmed that LPA levels are elevated in plasma from patients with ovarian cancer using the MS-based method. Data analyses are in progress to determine the specificity of the test. More clinical samples will be collected in the third year of the grant to further assess the sensitivity and specificity of the test.

Using the MS method, we have found that a number of other lysophospholipids, including alkyl-LPA, alkenyl-LPA, LPI, SPC and LPC are also elevated in ascites from patients with ovarian cancer, compared with ascites from patients with non-malignant diseases (3). The diagnostic, prognostic, and clinic management significance of these lipids is under investigation.

Importantly, we have recently identified the first receptors for SPC and LPC (4-7). These discoveries provide an intriguing opportunity and a novel approach to study the roles of SPC and LPC in ovarian cancer. In addition, we have found lysophospholipase D (Lyso-PLD) activity in ovarian cancer ascites. To target these receptors and lyso-PLD as an early intervention strategy is under investigation.

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APPENDICES

Journal articles:

1. Ni X, Huang KC, Zhang W, Wang Y, Ng SK, Mok SC, Berkowitz RS, Ng SW. Characterization of human kallikrein 6/protease M expression in ovarian cancer. Submitted.
2. Ho SM, Lau KM, Mok SC, Syed V. Profiling follicle stimulating hormone-induced gene expression changes in normal and malignant human ovarian surface epithelial cells. *Oncogene*. 2003, 22:4243-4256.

Characterization of human kallikrein 6/protease M expression in ovarian cancer

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ABSTRACT

Purpose: Kallikrein 6 (hK6, also known as protease M/zyme/neurosin) is a member of the human kallikrein gene family. We have previously cloned the cDNA for this gene by differential display and shown the overexpression of the mRNA in breast and ovarian primary tumor tissues and cell lines. To thoroughly characterize the expression of this kallikrein in ovarian cancer, we have developed a monoclonal antibody specific to hK6 and employed it in immunohistochemistry. Kallikrein 6 mRNA levels were determined by quantitative real-time reverse transcription-polymerase chain reactions. The expression was found elevated in most of the tumor samples. To investigate the mechanism of kallikrein 6 overexpression, we also performed genomic Southern analysis to determine if gene amplification is a mechanism for the elevated expression of kallikrein 6 in ovarian tumors .

Experimental Design: Specimens from 3 normal ovaries, 9 benign ovarian tissues, 18 borderline and 62 invasive ovarian tumors were analyzed.

Results: Kallikrein 6 was expressed in 67 of 80 cases of ovarian tumor tissues and there was a significant difference in the expression levels between normal and benign ovarian tissues and the borderline and invasive tumors ($P < 0.001$). There was no difference of expression level between different subtypes of tumors. More significantly, high level of kallikrein 6 expression was found in many early-stage and low-grade tumors, and elevated hK6 proteins were found in benign epithelia coexisting with borderline and invasive tissues, suggesting that overexpression of hK6 is an early phenomenon in the development of ovarian cancer. Quantitative real-time reverse transcription-polymerase chain reactions also showed elevated kallikrein 6 mRNA expression in ovarian tumors. Genomic Southern analysis of 19 ovarian tumor samples suggested that gene amplification is one mechanism for the overexpression of hK6 in ovarian cancer.

Conclusions: Our findings indicate that kallikrein 6 expression is significantly overexpressed in many ovarian tumors. As kallikrein 6 is also highly expressed in many early-stage ovarian tumors, kallikrein 6 may be involved in the early stages of cancer

development and has high potential as an ovarian tumor biomarker for early detection of the disease. Gene amplification is one mechanism for the overexpression of kallikrein 6 in ovarian cancer.

INTRODUCTION

Ovarian cancer is the fourth most common form of cancer in females in the United States and accounts for more than half of the deaths due to gynecological malignancy (1). Ovarian cancer of epithelial origin, which constitutes more than 90% of the disease, is the most lethal among all gynecological malignancies, with a 5-year survival rate of only 20% (1-4). The poor prognosis is mainly due to the lack of symptoms at the early stage of disease. At the time of diagnosis about 70% of patients have cancer cells already spread to the pelvic and abdominal viscera or developed distant metastasis (Stage III/IV) and are rarely curable. In contrast, the survival rate will exceed 90% at five years if the disease is detected in Stage I, when tumors are confined to the ovaries (5). Therefore, early detection and early intervention is critical to improve the clinical outcome of ovarian cancer patients.

Ovarian carcinogenesis is believed to be a multi-step process. To investigate the features of the earliest form of ovarian carcinoma, extensive pathological studies of grossly normal ovarian samples have revealed that high-grade serous ovarian carcinomas might develop *de novo* from surface epithelium and its inclusions, as well as from endosalpingeal tissues in the cortical stroma (6, 7). The coexistence of cytologically benign, borderline and malignant epithelium in some ovarian carcinomas and evidence of areas of histologic transition from benign to borderline or to malignant epithelium support the contention that many low-grade ovarian carcinomas, particularly those of the mucinous type, may arise from pre-existing benign or borderline lesions (8, 9). Despite these pathological findings, the molecular basis for the pathogenesis of epithelial ovarian tumors is largely unknown. Discovery of biomarkers that are present early in the abnormal endosalpingeal tissues or in benign or borderline lesions coexisting with malignant carcinomas may further our understanding of the mechanism of early ovarian carcinogenesis.

We have previously identified by differential display a cDNA sequence that is highly expressed in primary breast and ovarian tumor tissues and cell lines (10). The encoded

protein, which originally was named as protease M, shows strong homology to the human kallikrein (hK) family proteins (reviewed in 11, 12) and has a revised nomenclature of kallikrein 6 (*KLK6* for the gene and hK6 for the encoded protein). There are at least 15 human kallikrein genes that are co-localized as a cluster within a 300-kb region at chromosome 19q13.3-13.4. They also share significant similar genomic organization and homology at both the nucleotide and amino acid levels (12). All genes encode for putative serine proteases with conserved signal peptide sequence for secretion and catalytic triad residues in the appropriate positions. Of them, kallikrein 3 (hK3), or more commonly named as prostate-specific antigen (PSA), has gained prominence as the most valuable tumor marker and is currently used widely for the diagnosis, monitoring, and population screening for prostate cancer (13, 14). hK3 has also been reported to cleave insulin-like growth factor-binding protein-3, resulting in increased availability of insulin-like growth factor. This suggests that hK3 enzymatic activity may promote proliferation, migration, and metastasis of prostate cancer cells (15). It will be of interest to determine the functions and the potential of serum biomarker development for the other members of the kallikrein family. Indeed, recent studies have shown that the expression of many kallikreins including hK4, hK5, hK6, hK7, and hK8 has emerged as being related to breast, ovarian, and other human cancers (16-19). Further studies of these kallikreins in ovarian cancer and the development of detection tools may facilitate better understanding of this family of proteases in ovarian cancer and improve the prognosis of ovarian cancer patients.

In order to characterize the expression pattern of hK6 in different subtypes and stages of ovarian tumor tissues and to develop a tool for the potential use of measuring hK6 levels in patient serum, we have established a novel monoclonal antibody that has high specificity and reactivity to hK6. Here we report the utility of this monoclonal antibody in determining the expression pattern of different subtypes and stages of ovarian tumor tissues. In conjunction with the data of mRNA expression, we found that hK6 is highly expressed in various subtypes of ovarian tumor tissues, and is also present in early-stage tumors. We also observed high expression of hK6 in the apparently benign epithelia coexisting with borderline and invasive tumors in some samples. The elevated expression of hK6 in early-

stage and low-grade ovarian tumors may suggest that up-regulation of this protein may be an early event during ovarian cancer development and hK6 may have potential use as biomarkers for the early detection of ovarian cancer. The presence of high-quality monoclonal antibodies will be beneficial for the development of a standard screening method for large-scale population screening. We have also performed genomic Southern analysis to determine if gene amplification is one mechanism for the overexpression of hK6 in ovarian tumors.

MATERIALS AND METHODS

Biologic Specimens

All patient-derived biologic specimens were collected and archived under protocols approved by the Human Subjects Committee of the Brigham and Women's Hospital, Boston, MA. Ovarian tissues and cells were freshly collected from women undergoing surgery at the Brigham and Women's Hospital for a diagnosis of primary ovarian cancer or from control subjects having hysterectomy and oophorectomy for benign disease. All tumor tissues were collected from the primary ovarian sites and were confirmed histologically. Cultures of normal human ovarian surface epithelial (HOSE) cells were established by scraping the surface of the ovary and growing the recovered cells in a mixture of medium 199 and MCDB105 medium supplemented with 10% fetal calf serum (Sigma Chemical Co.) as described previously (20). For fresh-frozen sections, fresh specimens were embedded in Tissue Tek OCT medium (Miles, Inc.), snap-frozen in liquid nitrogen, and stored at -80°C until use.

Isolation of monoclonal antibodies specific to hK6

cDNA for hK6 was amplified using RT-PCR reaction with the primers 5'-CTGGAAATTCTTGGTGCATGGCGGACCC-3' and 5'-CTGTCTAGATCACTTGGCCTGAATGGTTT-3'. The fragment was restricted with *Eco*RI and *Xba*I and subcloned into the pMAL-c2X vector DNA (New England BioLabs)

that has been restricted with the same enzymes. The resulting construct was transformed into the *E. coli* strain TB1. The procedures for the induction of maltose binding protein (MBP)-hK6 recombinant fusion protein synthesis by IPTG (isopropyl thiogalactoside), preparation of crude extracts, and the purification of the recombinant protein over the amylose resin column were according to the manufacturer's recommendation. Kallikrein 6 protein was released from the fusion protein by Factor Xa digestion and purified by SDS-polyacrylamide gel electrophoresis. The antigen was injected into five female Balb/c mice in a series of three injections given at 3-week intervals. The first consisted of 75 µg per mouse in Freunds complete adjuvant, while the second and third injections were 50 µg per mouse in incomplete Freunds adjuvant. Mice were boosted with 50 µg of protein four days prior to fusion.

The spleen cells were fused with NS-1/Ag3 myeloma cells using polyethylene glycol, and plated out in HAT-selective growth medium. After 10 days, supernatants from each well were assayed by enzyme-linked immunosorbent assay (ELISA) and colonies showing reactivity were also tested by Western blotting. Specific antibody-secreting hybridomas were picked and transferred to separate wells in a 24-well plate and the specificity was further confirmed by ELISA and Western blotting. Positive lines were subsequently cloned by double-dilution and re-assayed. Positive clones were expanded and the IgG subclasses were determined by ImmunoPure isotyping kit from Pierce.

In-situ immunohistochemistry

For *in-situ* immunohistochemistry, 7 micrometer sections were cut from the paraffin archived tissues and mounted on Superfrost/Plus microscopic slides (Fisher Scientific), and incubated at 50 °C overnight. They were deparaffinized in xylene and rehydrated in graded ethanol. For antigen unmasking, sections were immersed in antigen unmasking solution (Vector Lab, Inc.) and boiled in microwave oven for 10 minutes. The tissues sections were then washed in phosphate buffered saline (PBS) and quenched in 0.2% H₂O₂ for 20 min. The sections were washed in PBS for 20 min., incubated with normal horse blocking serum for 20 min., and subsequently incubated overnight with anti-hK6 antibody. After incubation,

the sections were washed in PBS for 10 min., incubated with diluted biotinylated secondary horse anti-mouse antibody for 30 min., and washed again in PBS for 10 min. After washing, the sections were incubated with VECTASTAIN Elite ABC reagent (Vector Inc.) for 30 min., washed in PBS for 10 min., incubated in diaminobenzidine (DAB) solution for 5 min and washed in water for 10 min. The sections were counterstained with haematoxylin, dehydrated with an ascending series of alcohol, cleared in xylene and mounted in Permount (Fisher Scientific). The specificity of the staining was confirmed by preabsorbing the antibody with the purified hK6 protein for 2 hrs at 37 °C before applying to the sections. The results of immunohistochemistry were quantified using a semi-quantitative scoring system (21). The weighted score is obtained by multiplying the staining intensity score (3+, strong positive stain in most cells; 2+, moderate stain in cells; 1+, weak stain in cells; 0, no evidence of stain) and score for the percentage of positive cells (3+, most of cells stained; 2+, half of cells stained; 1+, few cells stained; 0, no cells stained). Representative photomicrographs were recorded by a digital camera (Optronics, Inc.).

Laser Capture Microdissection

Tissues stored in Tissue Tek OCT medium at -80 °C were sectioned at 7 micrometer in a cryostat (Leica, Inc.). Sections were mounted on uncoated glass slides and immediately fixed in 70% and 50% ethanol for 30 seconds each, stained with hematoxylin-eosin, dehydrated in alcohol solution of increasing concentration, and cleared in xylene for 5 minutes. After being air-dried for 3 minutes, the sections were laser microdissected using the PixCell II apparatus (Arcturus, Inc.).

Quantitative real-time Reverse Transcription Polymerase Chain Reaction

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to quantify the mRNA levels of kallikrein 6 in the tissue samples. Frozen tumor tissues were pulverized on dry ice and total RNA was isolated using TRIzol reagent (Invitrogen). For LCM dissected materials, RNA was extracted using a StrataPrep Total RNA miniprep kit (Stratagene). cDNA synthesis was performed with 50 ng of RNA samples using the TaqMan reverse transcription reagent kit (Perkin Elmer), and quantitative PCR

reactions were performed using a SYBR Green I kit, which contained 2 μ l of cDNA, 1x SYBR PCR buffer, 3 mM MgCl₂, 0.8 mM dNTP, and 0.025 U/ μ l AmpliTaq Gold (PE Applied Biosystems, Inc.). Real-time PCR primers for kallikrein 6 (forward primer: 5'-TCCTTCCCCGACTCAAGAAT-3'; reverse primer: 5'-TCCGCCATGCACCAACTTA-3') were designed using the PrimerExpress software (Perkin Elmer). Another PCR reaction using a set of primers for the housekeeping gene, cyclophilin A, was used to normalize for variances in input cDNA. The reactions were performed in an ABI PRISM 5700 Sequence Detector (PE Applied Biosystems, Inc.) with denaturation for 10 minutes at 95 °C followed by 40 PCR cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 1 minute. The threshold cycle (C_T) value for each reaction, reflecting the number of PCR cycles needed to give exponential amplification of kallikrein 6 amplicon, and the relative level of kallikrein 6 for each sample was calculated as described (22). At least duplicated PCR reactions were performed and the values averaged for each sample.

Statistical Analysis

All analyses were performed using MINITAB version 13 (MINITAB Inc.). One-way ANOVA is adopted to test whether the mean kallikrein 6 expression levels vary depending on the subtype, diagnosis, grade, and stage respectively. The relative measures of kallikrein 6 mRNA level were compared using log-transformed values. When an overall F-test was significant in the ANOVA, Fisher's pairwise comparisons were used to compare the mean kallikrein 6 levels among groups.

Establishment of SKOV3 cells that overexpress hK6 and immunostaining

Full-length cDNA encoding kallikrein 6 was produced by reverse transcription polymerase chain reaction using the primer set (Forward primer: 5'-GGCGGACAAAGCCCGATTGTTCC-3', reverse primer: 5'-GATCTCGAGTCAATCGTGATGGTGATGGTGATGCTGGCCTG AATGGTTTTGGATCC-3'). The PCR product was first cloned into the TOPO TA cloning vector, pCRII-TOPO (Invitrogen). The cDNA was then restricted from the vector by *Eco*RI and *Not*I digestion and cloned into Invitrogen T-Rex mammalian inducible vector

pcDNA6/TO/mycHis A at *Eco*RI and *Not*I sites. The resulting construct was transfected into a SKOV3 cell line that also expresses regulator protein TetR from an integrated plasmid, pcDNA6/TR (Invitrogen). Positive sublines were selected using antibiotics blasticidin and zeocin as described in the manufacturer's protocol. For immunostaining, the SKOV3 cell line that harboring the hK6-expressing construct was induced in the presence of 1 μ M of tetracycline, while the control is the same cell line treated with the solvent vehicle. These together with OVCA 429 and the immortalized HOSE cell line were fixed by adding 4% paraformaldehyde. The fixed cells were washed twice in PBS for 20 min., permeabilized in 0.2% Triton X-100 in PBS, washed again, incubated with normal horse blocking serum for 20 min., and subsequently incubated with anti-hK6 antibody at room temperature for 1 h. After incubation, the cells were washed in PBS for 10 min., incubated with diluted biotinylated secondary horse anti-mouse antibody for 30 min., and washed again in PBS for 10 min. After washing, color development was performed using the VECTASTAIN Elite ABC Kit (Vector Inc.) as described above.

Genomic Southern analysis

Frozen tumor tissues were pulverized on dry ice and genomic DNA was isolated according to the standard protocol (23). For Southern blot analysis, 5 μ g of genomic DNA was restriction digested with *Bam*HI and electrophoretically resolved on a 1% agarose gel. The genomic DNA was denatured and neutralized, transferred to nylon membrane (Amersham) according to the manufacturer's recommendation. Full-length kallikrein 6 cDNA was labeled with α -[³²P] dCTP using the multiprime labeling kit (Roche Molecular Diagnostics) and hybridized to the membrane using the standard protocol (23). Genomic DNA extracted from normal human ovarian surface epithelial primary cultures was used as control. Stringent hybridization and wash conditions were employed to ensure no cross-hybridization of the probe to other kallikrein genes. Hybridization signals were detected by autoradiography and quantified by a Bio-Rad BGS-700 densitometer.

Quantitative real-time Polymerase Chain Reaction for genomic DNA

Quantitative real-time polymerase chain reaction (qPCR) was used to quantify the gene copy number of kallikrein 6 gene for the tissue samples. 10 ng of tissue genomic DNA and two kallikrein 6 primer sets (5'-end primer set: 5'-ACCCTCCAGCCCATACCAAC-3' and 5'-ACATGGGAAACCACAGGCA-3'; and 3'-end primer set: 5'-GGACGCAAAGAAAGGGCAG-3' and 5'-CCACCTCGTGTCTGAGGACA-3') were used in the genomic qPCR reactions. Quantitative PCR reactions were performed using a SYBR Green I kit as mentioned for qRT-PCR. Primer sets for two single-copy genes, phosphatidylserine decarboxylase (5'-AGCAGAGCCACACAGCCTTC-3' and 5'-GGTGAATGTGGAACGGAAA-3') and DNA topoisomerase II beta (5'-CCTCATCCTTAGAGGCCCA-3' and 5'-GAGACCTAACCGGGAATCCG-3') were used to normalize the input DNA for different samples.

RESULTS

Isolation and characterization of monoclonal antibodies specific to kallikrein 6. In order to generate specific antibody for hK6, full-length cDNA for *KLK6* gene was cloned into maltose-binding protein (MBP) encoding pMAL-c2X vector as described in Materials and Methods. Fusion proteins were purified from the lysates of transformed *E. coli*. The hK6 protein was released from the fusion by Factor Xa digestion and used in the immunization of mice. Spleen cells harvested from one of the immunized mouse were fused with the myeloma cell line NS-1/Ag3. After screening for more than 1000 clones, one of the clones, 2D4, showed very high reactivity and specificity to hK6 protein. 2D4 does not cross-react with trypsin and other kallikrein members such as hK1 and hK3 (data not shown). As shown in Figure 1, the antibody stained positively the cytoplasm of a SKOV3 cell line that was induced to express hK6 by tetracycline (Figure 1a) but not in the uninduced cells (Figure 1b). The antibody also recognized the endogenous hK6 proteins present in the OVCA429 cell line (Figure 1c). The immortalized normal human ovarian surface epithelial

(HOSE) cells only stained negatively in the assay (Figure 1d). The IgG subclass of 2D4 was determined to be IgG2b by ImmunoPure isotyping kit from Pierce.

Characterization of hK6 expression in normal and tumor ovarian tissues. We employed 2D4 in immunohistochemical studies to determine the expression levels of hK6 in paraffin block sections of 3 normal ovarian tissues, 9 benign tissues, 18 borderline and 62 invasive ovarian tumors. The mean immunostaining scores in tissue sections from healthy ovary, benign ovarian tumor, borderline ovarian tumor, and invasive ovarian cancer were 0.0, 1.83, 3.42, and 3.71, respectively (Table 1). The overall F-test for the diagnostic groups was statistically significant ($P < 0.001$), indicating that mean immunostaining scores varies between diagnostic groups. The Fisher's pairwise comparison procedure was employed to compare the mean immunostaining scores at 5% level. The result shows that the healthy and benign tumors have significantly lower scores than the borderline and invasive tumors. Within the cancer (borderline and invasive) groups, there was no significant difference among histologic groups, as well as among different grades and stages (Table 1). Figures 1(e through j) show representative results of the hK6 staining in normal ovarian epithelium, stage I and stage III serous tumors, as well as mucinous, endometrioid, and clear cell subtypes of ovarian tumor tissues. While there was no positive staining for the normal ovarian epithelial and stroma cells, hK6 immunoreactivity was observed in the cellular membrane and cytoplasm of tumor cells in the cancer groups.

In particular, we observed that in some mucinous tumor samples, there was strong hK6 staining in some apparently benign epithelia similar to the coexisting borderline tumors (Figure 2a), or in borderline tumors coexisting with invasive tumors (Figure 2b). As it is believed that mucinous ovarian carcinomas may arise from pre-existing benign or borderline lesions, it is very likely that elevated hK6 expression coincides with the early stages of ovarian cancer development.

Besides the evaluation of protein expression, we also determined kallikrein 6 mRNA levels between healthy human HOSE primary cell cultures and borderline and invasive

tumors. For the borderline and invasive tumor samples other than serous subtype, tumor cells were microdissected from frozen sections by Laser Capture Microdissection (LCM) and total RNA was extracted from the captured tumor cells. The results in Table 2 confirmed the significant differences in kallikrein 6 mRNA expression between normal HOSE primary cell cultures and tumor tissues ($P < 0.001$). For comparisons between different subtypes of tumors, the F-test in the ANOVA was marginally significant ($P = 0.054$), indicating that the mean expression levels were not the same for different subtypes of tumors. The result of the Fisher's pairwise comparison further indicated that mixed tumors had significantly lower mRNA expression levels than other types of tumors. It can be seen from Table 2 that the mean kallikrein 6 mRNA levels are not significantly different among various stages and grades of borderline and invasive tumor samples.

Characterization of kallikrein 6 gene copy number in ovarian carcinomas. In order to determine if gene amplification is one mechanism in causing elevated hK6 expression, we have performed genomic Southern analysis for 19 ovarian carcinoma samples. The results are presented in Figure 3. Four out of 19 ovarian carcinomas exhibited higher copy number of kallikrein 6 gene. We also performed quantitative real-time PCR with the genomic DNA samples and the results were consistent with those of the genomic Southern analysis (Figure 3).

DISCUSSION

Because most ovarian cancer patients are diagnosed only at a late stage and have a dismal overall survival rate, there is a paramount need for better early detection methods. Although a number of tumor markers have been identified (24-27), a useful screening marker for ovarian cancer has not yet been clearly established. The most widely used marker, CA125, has shown merit in pilot screening studies and ovarian cancer management, but the sensitivity for early-stage disease before clinical detection remains questionable (28).

We have previously identified by differential display a cDNA that is highly expressed in ovarian tumor (10). Sequence analysis has shown that this novel protein belongs to the human kallikrein protein family. One member of the kallikrein family, prostate-specific antigen (PSA), has been successfully used in the diagnosis and clinical management of prostate cancer (13, 14). The high expression of hK 6 in ovarian tumor cells and its secretory nature suggests that this kallikrein may have potential as a novel biomarker for ovarian cancer detection. To develop a useful screening tool and to examine the clinical relevance of hK 6, we have developed a novel monoclonal antibody that has high specificity and reactivity to hK 6. This antibody does not react with other kallikrein members and it may be very valuable in evaluating the clinical potential of hK6.

The employment of the monoclonal antibody in immunohistochemistry of archived ovarian paraffin sections has shown the significant overexpression of hK 6 proteins in ovarian tumor cells compared with normal ovarian epithelium and benign diseases. There was no significant difference in hK6 expression among different subtypes, grades, and stages of ovarian cancer groups. However, we saw a slightly higher proportion of clear cell samples that overexpress hK6 (Table 1). High expression of hK6 was also observed in the borderline and early-stage invasive samples. In particular, we observed that in some mucinous tumor samples, there was strong hK6 staining in some apparently benign epithelia similar to the coexisting borderline tumors (Figure 2a), or in borderline tumors coexisting with invasive tumors (Figure 2b). Because it is believed that mucinous ovarian carcinomas may arise from pre-existing benign or borderline lesions (8, 9), up-regulation of hK6 expression may be an early event of cancer development and hK6 may have potential as a novel biomarker for early detection of ovarian cancer. In addition, mRNA levels were mostly consistent with the protein levels, suggesting that transcriptional regulation may be a mechanism that regulates the expression of hK6. Interestingly, many of the kallikrein genes are co-regulated by steroid hormones (29) and recent analysis of the genomic sequence of the kallikrein gene cluster has identified a minisatellite element that is present only in this region of chromosome 19 (30). Studies of the significance of this element and other potential sequences that regulate the expression of kallikrein genes may reveal the early

events of kallikrein up-regulation, particularly in relation to ovarian cancer development. Nevertheless, our genomic Southern analysis and genomic quantitative real-time PCR data have indicated amplification of kallikrein 6 gene in some ovarian tumor samples. As chromosome 19q13 is nonrandomly rearranged in many human solid tumors including pancreatic carcinomas, astrocytomas, thyroid tumors and ovarian cancers (31), gene amplification is likely another mechanism for the elevated expression of hK6 in ovarian tumors.

The functional roles of kallikrein 6 and many other kallikreins have not yet been established. As they are secreted serine proteases, their actions through the degradation of the extracellular matrix may facilitate tumor cell spread. Alternatively, they may be part of an enzymatic cascade pathway that involves enzyme activation followed by proteolysis (32). The establishment of functional recombinant proteins and kallikrein-expressing cell lines will facilitate functional studies and provide information about the possible roles of these kallikreins in aberrant cell growth and cell invasion.

In summary, we have developed a novel monoclonal antibody to confirm the high expression levels of hK6 in ovarian tumor cells than in normal ovarian epithelial cells. High expression of hK6 protein in coexisting benign, borderline and invasive ovarian tumors was observed. The examined tumors also expressed high levels of kallikrein 6 mRNA. Southern blot and quantitative real-time PCR using genomic DNA suggested that gene amplification is one mechanism for the high expression of kallikrein 6 in ovarian tumors. The development of high quality monoclonal antibodies facilitates the evaluation of biomarkers and allows further screening tool development for early detection of ovarian cancer.

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Table 1. Expression of hK6 in relation to histopathologic characteristics by immunohistochemical analysis

Characteristics	No. of Patients	Mean of Scores	P Value
All patients	92	3.35	
Diagnostic category			
Healthy	3	0.00	
Benign	9	1.83	
Borderline	18	3.42	<0.001
Invasive	62	3.71	
Histology of cancer			
Serous	44	3.44	
Mucinous	16	3.94	
Endometrioid	9	3.11	0.205
Clear Cell	4	5.00	
Mixed	7	4.14	
Tumor differentiation			
Borderline	18	3.42	
Grade 1	23	3.46	
Grade 2	7	4.07	0.792
Grade 3	29	3.64	
Stage			
I	24	3.48	
II	11	3.36	
III	31	3.61	0.965
IV	5	3.70	

Table 2. Human kallikrein 6 mRNA expression in ovarian tissues.

Characteristics	No. of Patients	Mean of Expression	P Value
All patients	59	3.61	
Diagnostic category			
Healthy	7	-0.76	
Borderline	5	4.48	< 0.001
Invasive	46	4.05	
Histology of cancer			
Serous	35	4.61	
Mucinous	6	3.00	
Endometrioid	5	4.67	0.054
Clear Cell	2	4.42	
Mixed	4	1.64	
Tumor differentiation			
Borderline	4	4.78	
Grade 1	5	4.04	
Grade 2	9	3.62	0.819
Grade 3	33	4.26	
Stage			
I	10	4.21	
II	4	3.16	
III	28	4.26	0.757
IV	7	3.66	

Expression is determined by quantitative real-time reverse-transcription PCR. For statistical analysis, natural logarithm was taken for the expression levels of KLK6 in tumor cells relative to normal HOSE cells.

LEGEND TO FIGURES

Figure 1. Use of monoclonal antibody 2D4 in immunostaining and immunohistochemistry.

Immunostaining of (a) tetracycline-induced hK6 in SKOV3 cell line; (b) the same cell line without tetracycline induction; (c) OVCA429 ovarian carcinoma cell line; (d) immortalized normal HOSE cells. Immunohistochemical staining for hK6 in (e) normal ovary; (f) endometrioid; (g) mucinous; (h) stage I clear cell; (i) stage I serous; and (j) stage III serous ovarian tumor tissues. Immunopositive cells were stained brown in the cytoplasm. To highlight the tumor cells, the slides were counterstained with hematoxylin (purple).

Figure 2. Elevated expression of hK6 in benign and borderline lesions of mucinous ovarian tumors.

Positive 2D4 staining in immunohistochemistry of (a) a mucinous case, showing both single stratified layer of benign (BN) epithelium and borderline (BOT) tumor component with pleomorphism; (b), a mucinous tumor showing positive 2D4 staining in both borderline (BOT) cells and invasive (INV) tumor components.

Figure 3. Genomic Southern analysis and quantitative real-time PCR of tumor DNA.

Equal amounts of 19 tumor DNA were restricted with *Bam*HI and electrophoresed on an agarose gel and Southern blot analysis was performed using the *KLK6* cDNA probe. The 5-kb and 3.2-kb bands of the *KLK6* gene are marked by arrows. Genomic DNA of a normal ovarian epithelial primary culture (HOSE) was used as control. Shown on the bottom are the results of the quantitative real-time PCR of the tumor samples relative to the normal HOSE cells.

Figure 1.

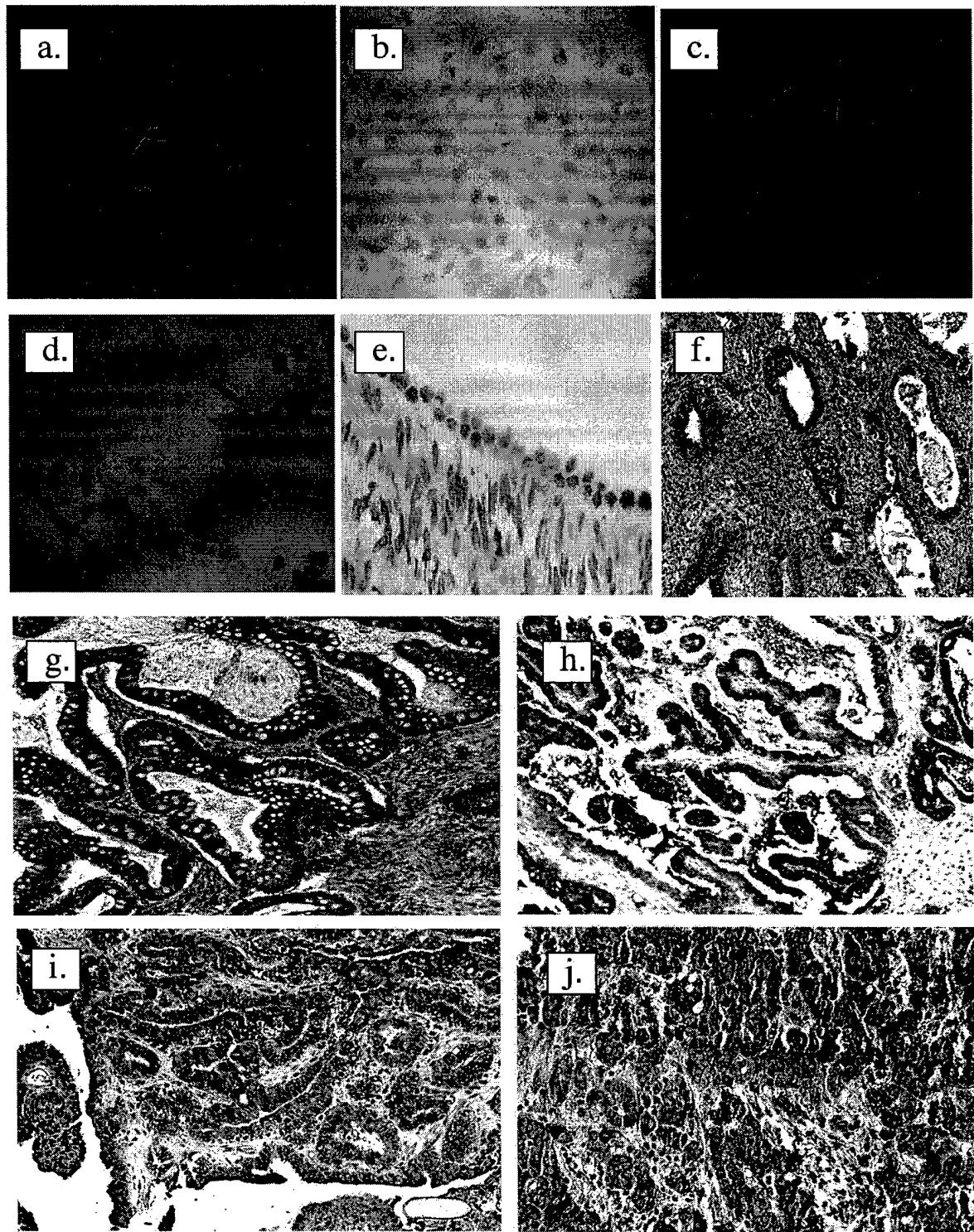


Figure 2.

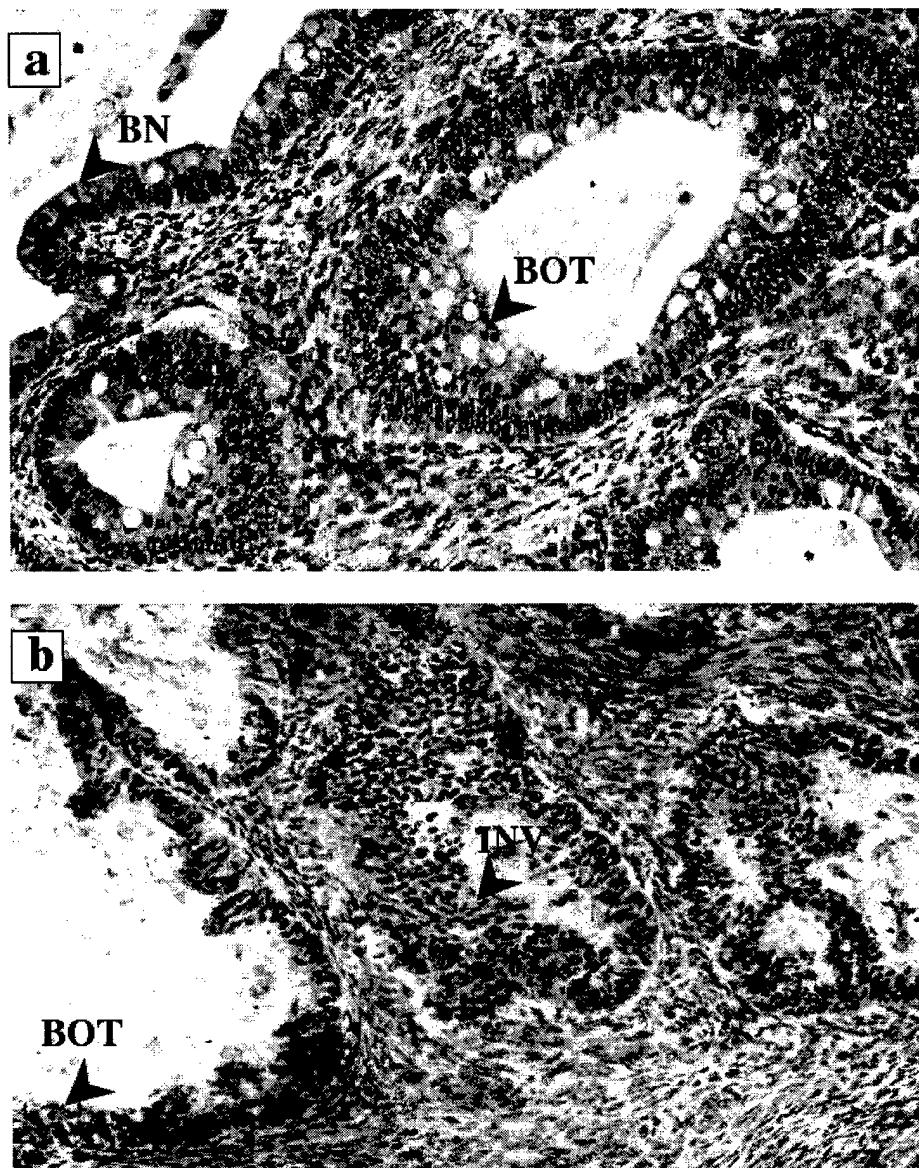
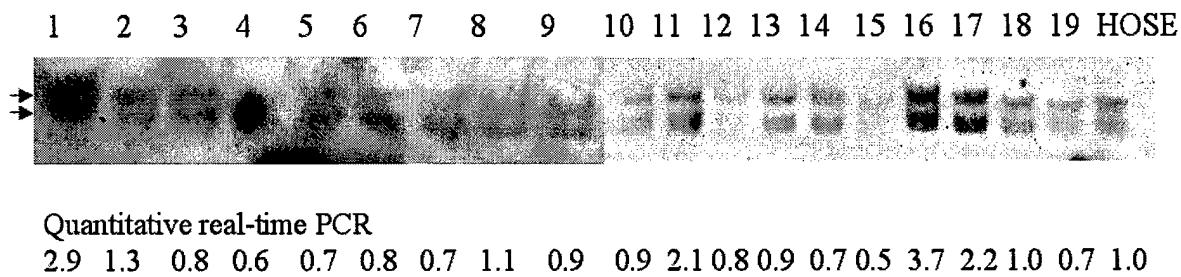


Figure 3.



Profiling follicle stimulating hormone-induced gene expression changes in normal and malignant human ovarian surface epithelial cells

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Epidemiological data have implicated the pituitary gonadotropin follicle stimulating hormone (FSH) as both a risk factor for and a protective agent against epithelial ovarian cancer. Yet, little is known about how this hormone could play such opposing roles in ovarian carcinogenesis. Complementary DNA microarrays containing 2400 named genes were used to examine FSH-induced gene expression changes in ovarian cancer (OC) and immortalized normal human ovarian surface epithelial (HOSE) cell lines. Two-way *t*-statistics analyses of array data identified two distinct sets of FSH-regulated genes in HOSE and in established OC cell lines established from patients (OVCA cell lines). Among the HOSE cell lines, FSH increased expression of 57% of the 312 genes and downregulated 43%. In contrast, FSH diminished expression of 92% of the 177 genes in the OVCA cell lines. All but 18 of the genes affected by FSH in HOSE cell lines were different from those altered in OVCA cell lines. Among the 18 overlapping genes, nine genes exhibited the same direction of change following FSH challenge, while the other nine showed discordance in response between HOSE and OVCA cell lines. The FSH-induced differential expression of seven out of nine genes was confirmed by real-time RT-PCR. Gene-specific antisense oligonucleotides (ODNs) were used to inhibit the expression of genes encoding GTPase activating protein (rap1GAP), neogenin, and restin in HOSE and OVCA cells. Antisense ODNs to neogenin and restin, but not an antisense ODN to rap1GAP, were effective in inhibiting OVCA cell growth, diminishing proliferating cell nuclear antigen expression, and increasing caspase 3 activities. Furthermore, the ODN to rap1GAP was further shown to be ineffective in altering migration properties of OVCA cell lines. HOSE cell proliferation was not affected by treatment with any of the antisense ODNs. In summary, gene profiling data reveal for the first time that FSH may exert different biological actions on OVCA cells than on HOSE cells, by differential regulation of a set of putative

oncogenes/tumor suppressors. Specifically, neogenin and restin were found to exhibit proproliferation/survival action on OC cells.

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Keywords: cDNA microarray; hormonal carcinogenesis; gene expression; GTPase activating protein (rap1GAP); neogenin; restin

Introduction

In the United States, ovarian cancer (OC) is the second most common cancer of the female reproductive system and the leading cause of death from gynecologic neoplasms (Holschneider and Berek, 2000). Each year, an estimated 23 100 US women are diagnosed with OC, and 14 000 die from the disease (Holschneider and Berek, 2000). Most ovarian tumors arise from the human ovarian surface epithelium (HOSE), which is a simple squamous-to-cuboidal mesothelium covering the ovary (Gillett *et al.*, 1999), or from invaginations of this lining into the superficial ovarian cortex to form cortical inclusion cysts (Feeley and Wells, 2001). During each menstrual cycle, the HOSE is ruptured at the site of ovulation and subsequently repaired via cell proliferation. The latter process is believed to be regulated by multiple reproductive hormones including gonadotropins (Auersperg *et al.*, 2001).

The pathogenesis of ovarian carcinoma remains unclear. Higher levels of gonadotropins after menopause are associated with an increased incidence of OC in perimenopausal and postmenopausal women (Hamilton, 1992; Godwin *et al.*, 1993; Hezisouer *et al.*, 1995; Konishi *et al.*, 1999; Rao and Slotman, 1999; Shushan *et al.*, 1996; Zheng *et al.*, 2000). Increased occurrence of OC has also been reported in women using gonadotropin-based fertility drugs (Whittemore *et al.*, 1992; Shushan *et al.*, 1996; Risch, 1998). Conversely, women who take acetaminophen as a pain-relief medicine have been found to have lower basal levels of gonadotropins and reduced OC risk (Cramer *et al.*, 1998). Clinical trials using LHRH agonist to suppress endogenous gonadotropins produced conflicting results, with some studies reporting no

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relevant beneficial effects (Erickson *et al.*, 1994; Emons *et al.*, 1996) and others suggesting increased long-term survival in individual patients cotreated with tamoxifen (Hofstra *et al.*, 1999). Furthermore, patients with OC, when compared to cancer-free patients, were found to have elevated levels of gonadotropins and ovarian hormones in their peritoneal fluid (Halperin *et al.*, 1999). Receptors for follicle-stimulating hormone (FSH) were detected in some OC/HOSE cell lines (Zheng *et al.*, 1996; Mandai *et al.*, 1997; Parrott *et al.*, 2001; Syed *et al.*, 2001) but were absent from others (Ala-Fossi *et al.*, 1999). Treatment of normal and malignant HOSE cells with gonadotropins, particularly FSH, demonstrated efficacy in stimulating cell proliferation in some cell lines (Zheng *et al.*, 2000; Syed *et al.*, 2001) but was either ineffective (Ala-Fossi *et al.*, 1999) or inhibitory (Ivarsson *et al.*, 2001) for other cell lines. Taken together, the aforementioned findings generally support the hypothesis that elevated gonadotropin levels, via enhancement of cell proliferation in the HOSE cell population, favor accumulation of genetic errors and neoplastic transformation (Konishi *et al.*, 1999; Holschneider and Berek, 2000; Feeley and Wells, 2001). Nevertheless, a portion of the literature argues against an association between high gonadotropin levels and increased OC risk.

In the present study, the gene expressions of two types of cell lines were compared to gain insight into the process of neoplastic transformation and the role of FSH: (1) normal immortalized HOSE cell lines that exhibited uniform epithelial-like morphology, were immunopositive for cytokeratins K7, K8, K18, and K19, immunonegative for vimentin, expressed no CA-125, and were nontumorigenic in nude mice (Taso *et al.*, 1995) and (2) OVCA cell lines derived from patients with late-stage serous ovarian adenocarcinomas (Bast *et al.*, 1981).

Although several studies have demonstrated the utility of microarray technology in identifying novel molecular markers for OC (Schummer *et al.*, 1999; Wang *et al.*, 1999; Ismail *et al.*, 2000; Ono *et al.*, 2000; Hough *et al.*, 2001; Shridhar *et al.*, 2001; Welsh *et al.*, 2001), this methodology has not been exploited in unraveling the role of hormones in ovarian carcinogenesis. We here employed a cDNA microarray containing 2400 named genes to profile gene expression changes induced by FSH in HOSE and OC cell lines in an effort to ascertain differential responses between normal and malignant HOSE cells to hormone stimulation. Results from this study have provided new insights into the role of FSH in ovarian carcinogenesis.

Results

Identification of genes whose expression was altered in all HOSE and OVCA cell lines following FSH treatment

Gene expression profiles in the three HOSE cell lines before hormone treatment were compared to those observed in the same lines after 5 days of FSH treatment

with individual microarray analyses. Similarly, microarray analyses were conducted to profile gene expression changes in the OVCA cell lines following FSH treatment. In this study, each cell line was microarrayed three times using three different mRNA samples. Gene expression levels in untreated cultures were plotted against those found in FSH-treated cell cultures as scatterplots (data not shown), and the Pearson correlation coefficient r for each comparison was calculated: $r=0.69$ for HOSE 642, $r=0.68$ for HOSE 6-8, $r=0.63$ for HOSE 12-12, $r=0.71$ for OVCA 420, $r=0.74$ for OVCA 429, and $r=0.53$ for OVCA 432. These values indicated that the majority of the 2400 genes showed no

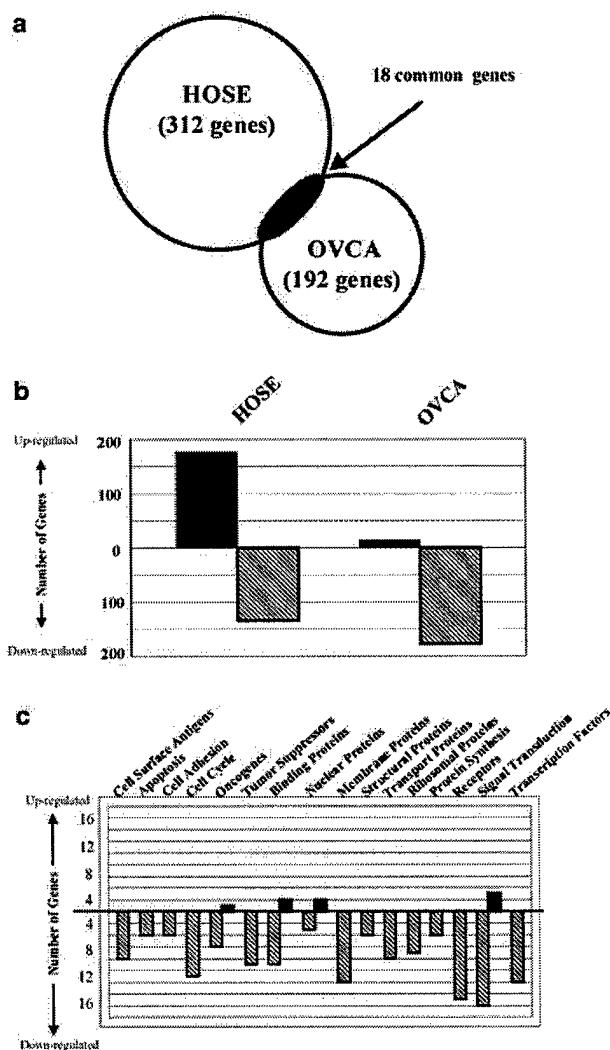


Figure 1 (a) Total number of genes altered in HOSE and OVCA cells by FSH. Common genes differentially regulated in both cell types are shown in black. (b) Total number of genes changed in HOSE and OVCA cells by FSH. Upregulated genes are shown by solid bars and downregulated genes are shown by hatched bars. (c) Categorized changes in gene expression in OVCA cells by FSH. Gene expression is upregulated in four listed categories (solid bars), while decreased expression is observed for all listed categories (hatched bars)

change in their levels of expression following FSH treatment. However, the sets of genes whose expression was altered in all three HOSE cell lines or in all three OVCA cell lines were identified by *t*-statistics analyses (Callow *et al.*, 2000). The probability, *P*-value, for each gene was also calculated to determine whether differential expression of a gene was due to FSH or to random or systematic variations. Genes with an absolute *t*-score above 2.0 and a *P*-value below 0.05 were considered differentially expressed with statistical significance in HOSE or in OVCA cell lines under the influence of FSH.

A total of 312 genes were differentially expressed in all three immortalized HOSE cell lines after FSH treatment, and 192 genes were identified as having been changed in all three OVCA cell lines after FSH exposure (Figure 1a). Interestingly, between these two sets of genes, only 18 were found in both sets, that is, exhibited altered expression in all six cell lines (normal immortalized and malignant) following FSH treatment.

Among the 312 genes whose expression changed following FSH treatment of HOSE cell lines, 177 (57%) genes were upregulated, and 135 (43%) genes were downregulated (Figure 1b). In marked contrast, of the 192 genes whose expression was affected by FSH in OVCA cell lines (Figure 1b), only 15 (8%) were upregulated and 177 (92%) were downregulated. The genes that were differentially regulated by FSH in the three OVCA cell lines could be categorized into 16 groups (Figure 1c). They included genes encoding cell surface antigens, oncogenes, tumor suppressors, binding proteins, membrane proteins, structural proteins, transport proteins, ribosomal proteins, receptors and transcription factors, as well as genes involved in apoptosis,

cell adhesion, cell cycle, protein synthesis, and signal transduction (Figure 1c).

Among the 18 genes whose expression was changed by FSH in all six cell lines, nine genes were found to be regulated in the same manner by the hormone in both HOSE and OVCA cells, with six genes showing enhanced expression and three exhibiting reduced expression after FSH treatment (Table 1). Nevertheless, the remaining nine genes showed discordance in FSH-regulated expression between HOSE and OVCA cell lines. In almost all cases, FSH stimulated gene expression in HOSE cell lines but caused reduced gene expression in OVCA cells. The fold-decrease or fold-increase as well as the *t*- and *P*-values for these 18 genes are listed in Table 1.

Comparison of gene expression between HOSE and OVCA cell cultures and confirmation of opposite direction of regulation by FSH in normal and malignant cell lines

Our microarray experiments identified a total of nine genes whose expression was upregulated by FSH in HOSE cell lines but downregulated by the hormone in OVCA cell lines. This set of genes was selected for further *post hoc* analyses by quantitative real-time RT-PCR to (1) determine whether they are differentially expressed between the normal and malignant cell lines, and (2) confirm the discordance in regulation by FSH. The nine genes were those encoding GTPase activating protein (rap1GAP), neogenin, restin, heat shock factor-2, protein phosphatase inhibitor-2, *N*-methyl-D-aspartate (NMDA) receptor 2A, ATP-dependent RNA helicase, carcinoembryonic antigen, and PLSTIRE for serine/threonine protein. Based on whether these genes

Table 1 Differential regulation of FSH-induced genes in normal and malignant ovarian surface epithelial cells

Accession #	Gene name	HOSE			OVCA		
		Fold	<i>t</i> -value-n	<i>N</i> -FSH- <i>p</i>	Fold	<i>t</i> -value-c	<i>C</i> -FSH- <i>p</i>
M29540	Carcinoembryonic antigen mRNA (CEA)	3.244716	23.87134	0.009078	0.685282	-35.4734	0.039546
U90277	<i>N</i> -methyl-D-aspartate receptor 2A subunit precursor	2.084552	12.57861	0.033058	0.727395	-46.4704	0.035336
M65217	Heat shock factor-2 (HSF2)	1.968372	16.23115	0.029065	0.716839	-735.446	0.002384
X64838	mRNA for restin	1.889393	11.84889	0.041609	0.7655	-119.786	0.016903
U72391	Neogenin	1.866551	33.35391	0.01645	0.623439	-642.155	0.002055
X78873	mRNA for inhibitor 2 gene	1.819762	14.46826	0.037478	0.709252	-65.2142	0.024452
X66365	mRNA PLSTIRE for serine/threonine protein kinase	1.783692	127.1692	0.004943	0.583924	-23.6557	0.044172
M64788	GTPase activating protein (rap1GAP)	1.501961	32.06109	0.028443	0.645275	-26.0102	0.04672
AB001636	mRNA for ATP-dependent RNA helicase #46	1.39381	259.7478	0.004817	0.586826	-29.401	0.036687
AF026548	Branched chain alpha-ketoacid dehydrogenase kinase precursor	0.770407	-91.6624	0.022189	0.862499	-169.876	0.020122
D38496	mRNA for LZTR-1	0.734898	-57.7585	0.029768	0.639535	-50.1594	0.025552
AJ000519	mRNA for ubiquitin-conjugating enzyme UbcH7	0.684834	-28.1945	0.04826	0.663968	-491.969	0.002997
U65928	Jun activation domain binding protein	0.624973	-24.6122	0.046704	0.542119	-20.7551	0.045539
D56495	mRNA for Reg-related sequence derived peptide-2	0.50544	-501.03	0.002006	0.778689	-433.344	0.005133
D79205	mRNA for ribosomal protein L39	0.438198	-387.784	0.002279	0.410904	-173.726	0.004815
Y14736	mRNA for immunoglobulin kappa light chain	6.677026	2.144288	0.036619	1.790089	13.1657	0.042087
M23725	M2-type pyruvate kinase	6.479164	3.546475	0.023901	3.595391	3.591517	0.046309
X06256	mRNA for fibronectin receptor alpha subunit	2.428838	8.182007	0.037962	1.355123	36.57792	0.03455

This table shows the effect of FSH on gene expression in normal (n) HOSE and malignant (c) OVCA cells. FSH-induced fold increases or decreases of HOSE and OVCA cells are shown in columns 3 and 6, respectively. *t* values of FSH-treated normal cells (n) and cancer cells (c) are shown in columns 4 and 7, respectively. *P* values of FSH-treated normal cells (n) and cancer cells (c) are shown in columns 5 and 8, respectively.

are overexpressed, underexpressed, or equally expressed between HOSE and OVCA cell lines, we have grouped them into three groups for data presentation.

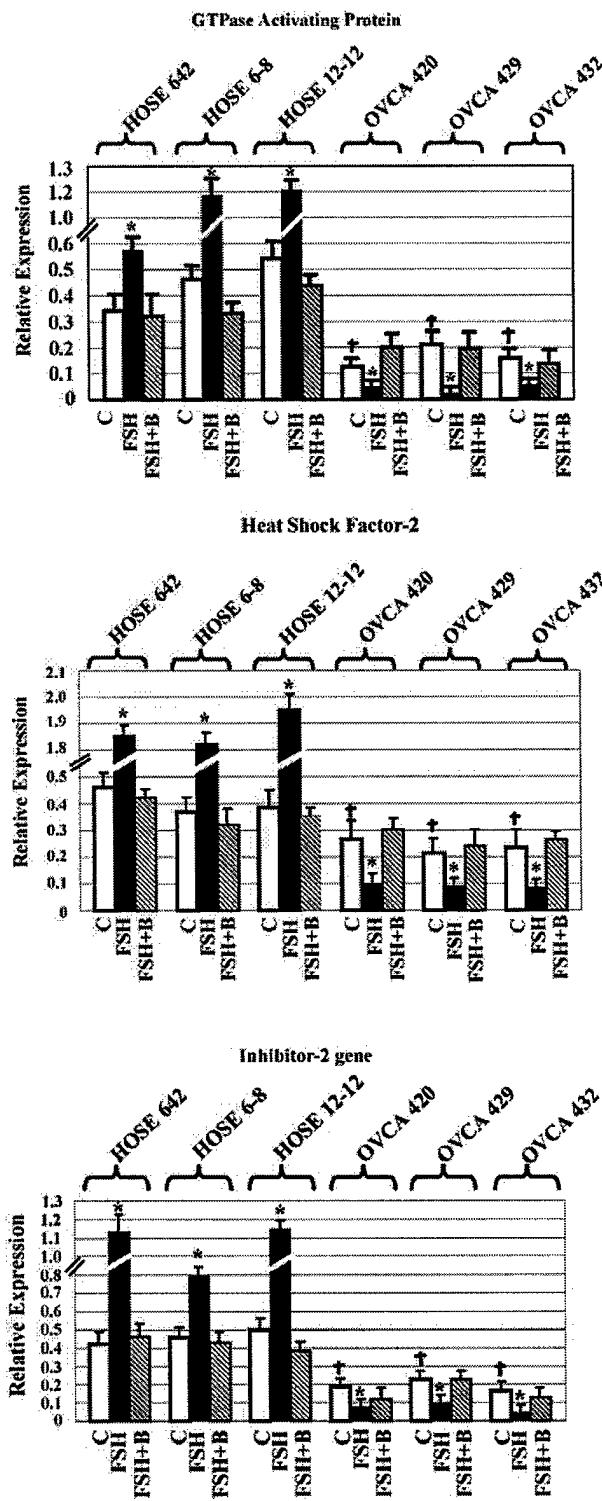
Genes encoding GTPase activating protein (rap1GAP), heat shock factor-2, and protein phosphatase inhibitor-2 were underexpressed in OVCA cell lines compared to HOSE cell lines, and their regulation by FSH exhibited discordance between HOSE and OVCA cell lines

Real-time RT-PCR demonstrated lower levels of expression of genes encoding rap1GAP, heat shock factor-2, and protein phosphatase inhibitor-2 in OVCA cell lines compared to immortalized normal HOSE cell lines (Figure 2). Marked increases in rap1GAP, heat shock factor-2, and protein phosphatase inhibitor-2 gene expression were observed in the three HOSE cell lines exposed to FSH, while gene expression was reduced in all OVCA cell lines by FSH, hence confirming the pattern of gene expression detected by microarray analyses. The effects of FSH on the expression of these three genes were hormone-specific; treatment of HOSE or OVCA cells with the PKA inhibitor H89 abolished the FSH-induced changes in all cases. Exposure of cell lines to PKA inhibitor alone caused no effect on cell proliferation (results not shown).

Genes encoding neogenin and restin were overexpressed in OVCA cell lines compared to HOSE cell lines, and their regulation by FSH exhibited discordance between HOSE and OVCA cell lines

Post hoc analyses by quantitative real-time RT-PCR demonstrated higher levels of expression of genes encoding neogenin and restin in OVCA cell lines compared to HOSE cell lines (Figure 3). Significant elevations in neogenin and restin gene expression were observed in the three HOSE cell lines exposed to FSH,

Figure 2 Real-time RT-PCR analysis of rap1GAP, heat shock factor-2, and protein phosphatase inhibitor-2 mRNAs in normal immortalized HOSE cell lines (HOSE 642, HOSE 6-8, and HOSE 12-12) and OC cell lines (OVCA 420, OVCA 429, and OVCA 432). Cells were treated as described in Material and methods, with FSH (10^{-8} M) in the absence (black bar) or presence of PKA inhibitor, H89 (10^{-5} M, diagonal hatched bar). The control cells were treated with vehicle (open bar). To amplify rap1GAP, the primers used were TGGACT-CAGAGGGAAACAAAGC (forward) and TGTAGCAGACATGCC-CAGAG (reverse). To amplify heat shock factor-2, the primers used were GACCCAGATCTCCTGGTTGA (forward) and CAA-GAAGTCGAAAGGCGGTA (reverse) and for phosphatase inhibitor-2 they were ATCTTGGCGACGTATCATCC (forward) and TACTTTGGCTCCAAGCCTTC (reverse). The intensity of PCR product was normalized to the intensity of GAPDH and shown in the figure. The data are shown as a mean of two experiments with triplicate samples and are represented as mean \pm s.e.m. Statistically significant FSH-induced decreases/increases in gene expression, compared to non-FSH-treated cells, are indicated by * $P < 0.05$. A statistically significant ($P < 0.05$) difference between untreated normal HOSE cells and untreated OVCA cells is indicated by T. C, control; FSH, follicle stimulating hormone; FSH+B, follicle-stimulating hormone + blocker of PKA



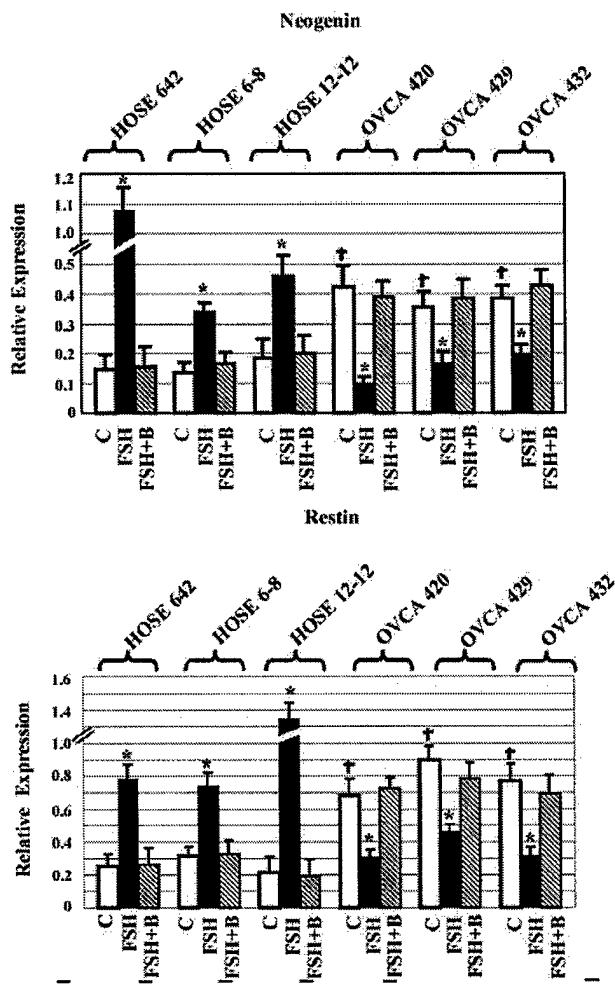


Figure 3 Real-time RT-PCR analysis of neogenin and restin mRNAs in normal immortalized HOSE cell lines (HOSE 642, HOSE 6-8, and HOSE 12-12) and OC cell lines (OVCA 420, OVCA 429, and OVCA 432). Cells were treated with FSH (10^{-8} M) in the absence (black bar) or presence of PKA inhibitor H89 (10^{-5} M, diagonal hatched bar). The control cells were treated vehicle (open bar). To amplify neogenin, the primers used were AAACCAACATCCCAGCAAC (forward) and AGTCACATCCTTGGTGGAG (reverse), and for restin the forward primer was TGCTGGACACAGAGGACAAG and the reverse primer was TGCAATTGTTGCTTGGTGT. The intensity of PCR product was normalized to the intensity of GAPDH and shown in the figure. The data are shown as a mean of two experiments with triplicate samples and are represented as mean \pm s.e.m. Statistically significant FSH-induced decreases/increases in gene expression, compared to non-FSH-treated cells, are indicated by * $P < 0.05$. A statistically significant ($P < 0.05$) difference between untreated normal HOSE cells and untreated OVCA cells is indicated by T. C, control; FSH, follicle-stimulating hormone; FSH+B, follicle-stimulating hormone + blocker of PKA

while expressions of both genes were reduced in OVCA cell lines by FSH exposure (Figure 3). The real-time RT-PCR findings confirmed the pattern of gene expression detected by microarray analyses. The effects of FSH on HOSE and OVCA cell lines were specific; cotreatment of cell cultures with H89 abolished the

FSH-induced alteration in gene expression (Figure 3). PKA inhibitor alone exerted no effect on cell proliferation in these cell lines (results not shown).

Genes encoding NMDA receptor 2A and ATP-dependent ATP helicase were expressed at comparable levels in HOSE and OVCA cell lines, and their regulation by FSH showed discordance between HOSE and OVCA cell lines

No significant differences were found in basal expression levels of NMDA receptor 2 (Figure 4), ATP-dependent

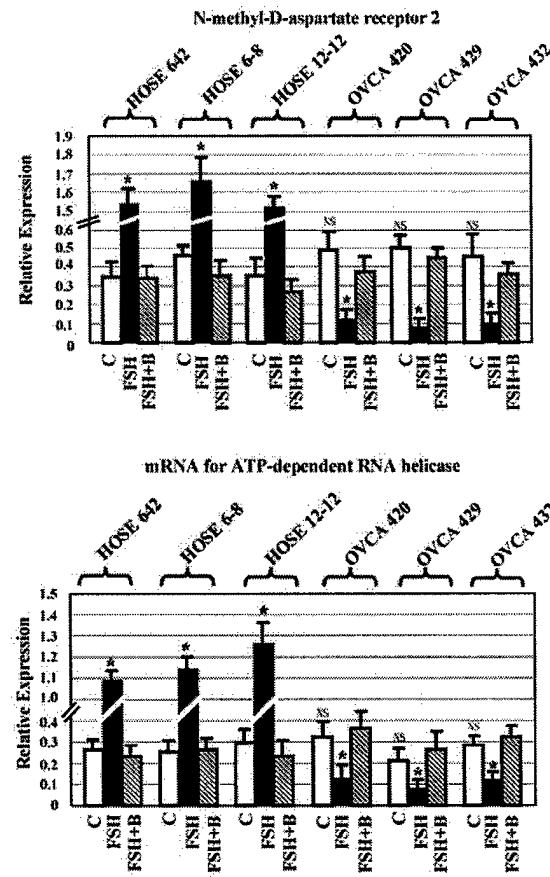


Figure 4 Real-time RT-PCR analysis of *N*-methyl-D-aspartate receptor-2 and ATP-dependent RNA helicase mRNAs in normal immortalized HOSE cell lines (HOSE 642, HOSE 6-8, and HOSE 12-12) and OC cell lines (OVCA 420, OVCA 429, and OVCA 432). Cells were treated with FSH (10^{-8} M) in the absence (black bar) or presence of PKA inhibitor H89 (10^{-5} M, diagonal hatched bar). The control cells were treated vehicle (open bar). The primers for amplification of *N*-methyl-D-aspartate receptor-2 were GGTCTGGAGGACAGCAAGAG (forward) and GGACAGTACGATGCCGTGA (reverse), and for ATP-dependent RNA helicase the primers were GTCCTTCAGCTCCCTGTTGG (forward) and ACTCATTGCAAGCCACTCTCC (reverse). The intensity of PCR product was normalized to the intensity of GAPDH and shown in the figure. The data are shown as a mean of two experiments with triplicate samples and are represented as mean \pm s.e.m. Statistically significant FSH-induced decreases/increases in gene expression, compared to non-FSH-treated cells, are indicated by * $P < 0.05$. Differences between untreated normal HOSE cells and untreated OVCA cells that are not statistically significant are denoted by NS. C, control; FSH, follicle-stimulating hormone; FSH+B, follicle-stimulating hormone + blocker of PKA

RNA helicase (Figure 4), carcinoembryonic antigen (not shown), and PLSTIRE for serine/threonin protein (not shown) mRNAs between normal immortalized HOSE and OVCA cells. Messenger RNA expression levels of NMDA receptor 2A (Figure 4) and ATP-dependent RNA helicase (Figure 4) were elevated in HOSE cell lines following FSH treatment, whereas significant decreases in their expression were observed in OVCA cell lines following FSH exposure (Figure 4). The PKA inhibitor H89 blocked the FSH-induced alterations in NMDA receptor-2 and ATP-dependent RNA helicase gene expression in both HOSE and OVCA cell lines.

However, we were unable to demonstrate differential expression of carcinoembryonic antigen or PLSTIRE for serine/threonin protein gene expression after treatment of HOSE or OVCA cells with FSH or FSH plus H89 (data not shown). Therefore, the differential expression detected by the microarray analyses for these two genes might represent false-positive results of microarray analyses.

Antisense ODN-mediated downregulation of neogenin and restin mRNA levels are associated with reduced cell growth in OVCA cell lines, but not in HOSE cell lines

HOSE and OVCA cell lines were exposed to gene-specific sense and antisense ODNs for neogenin, restin, and rap1GAP. Transcription of all three genes was downregulated in a dose-dependent manner in HOSE and OVCA cell lines exposed to their respective antisense ODNs (Figure 5a, b), while exposure of cell lines to the corresponding sense+antisense ODN mixtures produced no effects. In parallel, OVCA cell lines treated with antisense ODNs for neogenin or restin, but not for rap1GAP, exhibited a marked dose-dependent reduction in cell growth as measured by direct cell counts, MTS assay (Figure 5d), and PCNA mRNA expression (Figure 6a), when compared to cell cultures either not exposed to ODNs or to a combination of sense+antisense ODNs. In contrast to responses observed in OVCA cell lines, exposure of HOSE cell lines to antisense and combined sense+antisense ODNs of neogenin, restin, and rap1GAP caused no reduction in cell growth (Figure 5c). In order to provide evidence

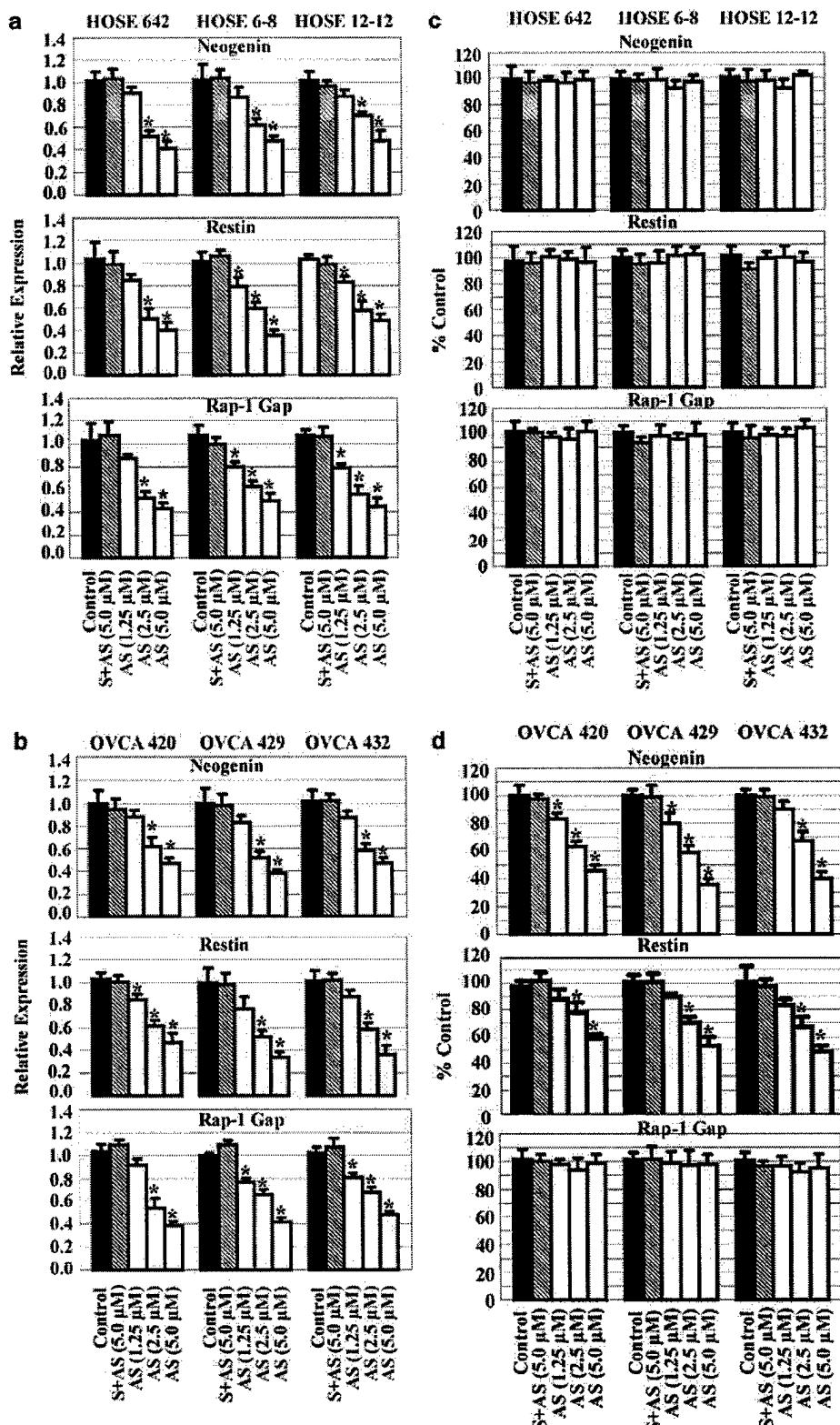
that the decreased numbers of cells in antisense-treated OVCA cell lines were because of induction of apoptosis, caspase 3 activity was measured (Figure 6b). Treatment of OVCA cell lines with antisense ODNs of restin or neogenin resulted in significant, dose-dependent increases in caspase 3 activity; no effects were produced by treatment with sense+antisense ODNs (Figure 6b). Treatment of OVCA cell cultures with antisense or sense+antisense ODNs of rap1GAP induced no changes in either cell number (Figure 6a) or caspase 3 activity in OVCA cell lines (data not shown).

Since antisense ODNs to rap1GAP produced no reduction in cell number in OVCA cell cultures, we performed a Matrigel Invasion Assay to test whether it might affect cell migration properties in these cell lines. OVCA cell lines treated with antisense ODNs of rap1GAP showed no discernable differences in their migration efficiencies through Matrigel.

Discussion

In the present study, we used cDNA expression array analysis to identify FSH-regulated genes in three immortalized normal HOSE cell lines and in three OVCA cell lines. One major challenge facing cDNA microarray studies is the appropriate use of algorithms in analysing results. Many studies used two- or three-fold up- or downregulation as arbitrary cutoffs to identify genes of interest (Lee *et al.*, 1999, 2001; Cao *et al.*, 2001). The major drawback of this approach is that genes that exhibited changes of less than 200–300% would not be recognized as differentially expressed. The approach presents a bias towards genes with higher degrees of differential expression and will miss genes that show consistent but smaller degrees of changes. Other published studies used pairwise comparisons of fold changes to set cutoffs (Lee *et al.*, 1999, 2001; Cao *et al.*, 2001). This statistical method provides some statistic power but has limitations related to its inability to take into consideration variations among the replicates. The *t*-statistics used in this study is a more robust algorithm that takes into consideration individual variations of the repeats (Callow *et al.*, 2000).

Figure 5 Effect of antisense ODNs of neogenin, restin, and rap1GAP on growth and mRNA expression of HOSE and OVCA cells. Cultured HOSE and OVCA cells treated with LipofectAMINE PLUS alone (control), sense and antisense ODNs (5 μ M sense + 5 μ M antisense), or different concentrations of ODNs (1.25, 2.5 and 5 μ M) for 48 h. (a) Dose-dependent effect of neogenin, restin, and rap1GAP antisense treatment on HOSE cells expression of neogenin, restin, and rap1GAP mRNA. LipofectAMINE PLUS alone (control), sense and antisense ODNs (5 μ M sense + 5 μ M antisense), or different concentrations of ODNs (1.25, 2.5, and 5 μ M). (b) Dose-dependent effect of neogenin, restin, and rap1GAP antisense treatment on OVCA cells expression of neogenin, restin, and rap1GAP mRNA. LipofectAMINE PLUS alone (control), sense and antisense ODNs (5 μ M sense + 5 μ M antisense), or different concentrations of ODNs (1.25, 2.5, and 5 μ M). (c) Cell growth was quantified by MTS assay. Sequence-specific, dose-dependent effect of neogenin, restin, and rap1GAP antisense treatment on HOSE (HOSE 642, HOSE 6-8, and HOSE 12-12) cell growth. Control (LipofectAMINE PLUS alone), sense and antisense ODNs (5 μ M sense + 5 μ M antisense), or different concentrations of ODNs (1.25, 2.5, and 5 μ M). Results represent mean \pm s.e.m. of triplicate samples, expressed as a percentage of cells treated with LipofectAMINE PLUS alone. (d) Sequence-specific, dose-dependent effect of neogenin, restin, and rap1GAP antisense treatment on OVCA (OVCA 420, OVCA 429, and OVCA 432) cell growth. Control (LipofectAMINE PLUS alone), sense and antisense ODNs (5 μ M sense + 5 μ M antisense), or different concentrations of ODNs (1.25, 2.5, and 5 μ M). Statistically significant decreases in gene expression/cell growth compared to that seen in control cells are indicated by * P <0.05. Results represent mean \pm s.e.m. of triplicate samples, expressed as a percentage of cells treated with LipofectAMINE PLUS alone



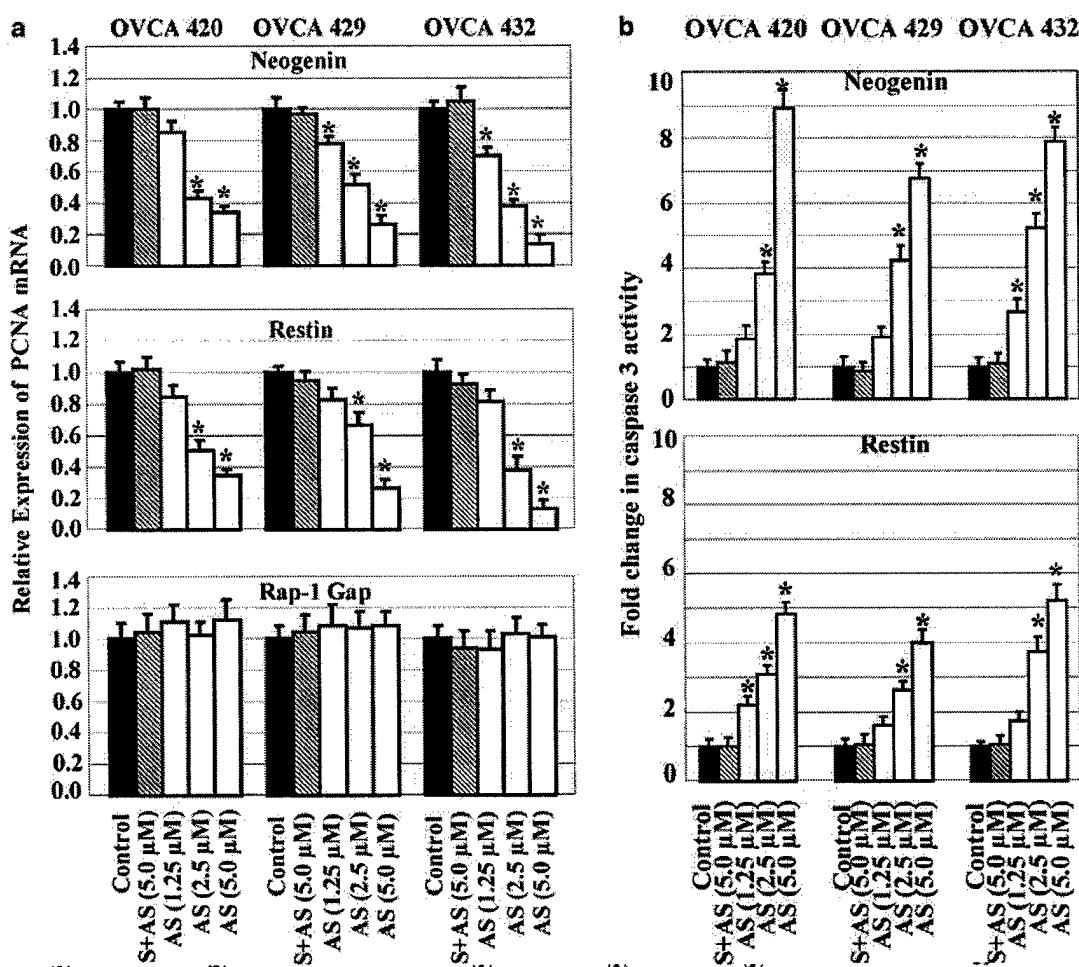


Figure 6 Expression of cell proliferation marker PCNA and apoptosis marker caspase 3 in OVCA cell lines. (a) Dose-dependent effect of neogenin, restin, and rap1GAP antisense treatment on OVCA cells PCNA mRNA expression. The primers for amplification of PCNA were GGCCTGAAACCTCACCAAGTAT (forward) and TCTCCCATATCCGCAATT (reverse). LipofectAMINE PLUS alone (control), sense and antisense ODNs (5 μM sense + 5 μM antisense), or different concentrations of ODNs (1.25, 2.5, and 5 μM). (b) Dose-dependent effect of neogenin and restin antisense treatment on OVCA cells caspase 3 activities. Enzyme activities of cell lysates toward tetrapeptide caspase substrate DEVD-pNA in OVCA (OVCA 420, OVCA 429, and OVCA 432) are shown in the figure. The caspase activity is expressed as fold to untreated controls and is represented as the means of two experiments performed in triplicate. Error bars represent the mean \pm s.e.m. LipofectAMINE PLUS alone (control), sense and antisense ODNs (5 μM sense + 5 μM antisense), or different concentrations of ODNs (1.25, 2.5, and 5 μM). Statistically significant changes in gene expression/caspase activity compared to that seen in control cells are indicated by $*P < 0.05$.

Using this approach, we were able to identify confidently gene expression changes smaller than onefold.

The principal finding of this investigation was that the gonadotropin apparently regulated one set of genes (total = 312) in the HOSE cell lines and an almost entirely different set of genes (total = 192) in the OVCA cell lines. It is also worth mentioning that only 18 genes were common to both gene sets. These findings strongly suggest that FSH exerts very different biological effects on normal HOSE cells than it does on OC cells. Among the 312 genes affected by FSH in the three HOSE cell lines, nearly half were upregulated and the other half downregulated. In sharp contrast, FSH caused downregulation of about 92% of the 194 genes affected by the hormone in the OVCA cell lines. Furthermore, it was of interest that most of the genes reported to be affected by FSH in our study have never been identified as FSH-

regulated genes in the past. Between the two sets, only 18 genes were commonly affected by FSH in all six (three HOSE and three OVCA) cell lines, and they fell into two categories. The first category contains nine genes whose expression was up- or downregulated by FSH in the same direction between HOSE and OVCA cell lines. We believe these genes are normally under FSH regulation and may have less relevance to ovarian carcinogenesis. In contrast, the other category of genes, whose expression exhibited the opposite direction of change, dependent on whether the cell type being challenged by FSH was normal or malignant, may have high significance to ovarian carcinogenesis. For this reason, we have focused our *post hoc* analyses only on the second category of genes in an attempt to demonstrate definitively the discordance in FSH regulation between normal and malignant HOSE cell lines.

One of the nine genes upregulated by FSH in HOSE cells but downregulated by the hormone in OVCA cells is rap1GAP, a purported tumor suppressor gene (Tsukamoto *et al.*, 1999). It is a member of the RAS family of small GTPase and is mapped to chromosomal region 1p36.1-35. rap1GAP has been shown to be activated by cAMP and phosphatase C pathways and is likely to be associated with adhesion, cellular growth, and/or differentiation (Altschuler *et al.*, 1995; Vossler *et al.*, 1997; Tsukamoto *et al.*, 1999). We demonstrated a loss of expression of this putative tumor suppressor in OVCA cells when compared to HOSE cells. These results are in accordance with the premise that rap1GAP is a tumor suppressor in OC cells. Furthermore, the reduction of rap1GAP mRNA expression in OVCA cell lines exposed to FSH agrees with the hypothesis that FSH is a risk factor for OC. Intriguingly, we found marked upregulation of rap1GAP expression in HOSE cell lines following FSH exposure. Since rap1GAP is required for the adherence and spreading of cells (Wani *et al.*, 1997), it is logical to speculate that after ovulation, the normal ovarian surface epithelium rapidly initiates the wound-healing process, which may involve FSH-induced upregulation of rap1GAP. The fact that antisense ODN against rap1GAP has no effect on the growth of HOSE or OVCA cells agrees with the known function of rap1GAP: that this molecule controls cell motility and focal adhesions rather than cell proliferation (Itoh *et al.*, 1999). However, since antisense ODN to rap1GAP showed no effect on OVCA cell migration through Matrigel, it remains to be determined whether this putative tumor suppressor gene may have effects other than regulation of cell proliferation and migration in these OC cell lines. We have preliminary data to show that rap1GAP may affect invasiveness of SKOV3, a more aggressive OC cell line.

Another gene that is underexpressed in OVCA cells when compared to HOSE cells is heat shock factor-2. Its expression is stimulated by FSH in HOSE cells but inhibited in OVCA cells. It has been shown that exposure of cells to stresses such as heat shock and oxidative injuries causes an imbalance in protein metabolism, to which the cells respond by co-expressing a family of heat shock factors (HSF1, HSF2, HSF3, and HSF4). Unlike other HSFs, HSF-2 is not activated in response to heat or most other forms of cellular stresses (Marimoto *et al.*, 1994) but is activated when the ubiquitin-proteasome pathway is inhibited (Mathew *et al.*, 1998). The downstream effect of HSF-2 is the induction of an array of heat shock genes that protect the integrity of the protein synthesis, folding, assembly, and degradation machinery (Marimoto *et al.*, 1994). Members of the heat shock protein family have been implicated in cancer (Fuqua *et al.*, 1994; Conroy and Latchman, 1996). The FSH-induced upregulation of heat shock factor-2 in normal HOSE cells may again be related to the wound-healing process after ovulation. However, in contrast, heat shock factor-2 expression is diminished in OVCA cells and further downregulated by FSH exposure. Reduction in its expression would

undoubtedly diminish the potential for OVCA cells to maintain protein fidelity during environmental or physiological stress. Further reduction in its expression by FSH likely exacerbates the adverse situation. Together, these may form an epigenetic basis of tumor progression.

Similar to rap1GAP and heat shock factor-2, protein phosphatase inhibitor-2 is underexpressed in OVCA cell lines compared to HOSE cell lines. FSH stimulated its expression in HOSE cells but inhibited expression in OVCA cells. Many physiological processes in mammalian cells are controlled by reversible protein phosphorylation, which is maintained by a rapid and precise regulation of the activities of various kinases and phosphatases (Oliver and Shenolikar, 1998). The activation of kinases is coordinated within the cell by the inhibition of phosphatases via phosphatase inhibitors (Oliver and Shenolikar, 1998). *In vitro* studies suggested that inhibitor-2 inhibits protein phosphatase activity and promotes a rapid and effective refolding of protein, yielding an active enzyme (Oliver and Shenolikar, 1998). In normal HOSE cells, FSH serves as a potent stimulator of inhibitor-2, which may be required during ovulation or postovulation HOSE cell function. On the other hand, a strong link between protein phosphatase inhibitor-2 and tumorigenesis and/or viral transformation of cells has been suggested (Cohen, 1989; Wera and Hemmings, 1994). In undifferentiated leukemias, phosphatase inhibitor-2 has been demonstrated to inhibit protein phosphatase-2 and accounts for enhanced growth of leukemic cells (Li *et al.*, 1996). In our study, OVCA cell lines appeared to express lower levels of phosphatase inhibitor-2, which was further diminished by FSH. Thus, OVCA cells may have higher growth potentials that could be further enhanced by FSH-mediated downregulation of protein phosphatase inhibitor-2.

NMDA receptor belongs to the family of ionotropic glutamate receptors. The NMDA receptor activation occurs when either glutamate (Glu) or NMDA and glycine (Gly) bind to the receptor molecule. A channel within the receptor complex enables molecules to cross the cell membrane and is blocked by magnesium (Mg). When Mg is removed from the channel and the receptor is activated, calcium (Ca^{2+}) and sodium (Na^+) ions enter the cell and potassium ions (K^+) leave. This receptor mediates alterations in intracellular calcium levels and thereby regulates a variety of signaling pathways, ranging from localized acute effects on receptor channel activities to long-term effects on gene transcription. A study has also established a link between downregulation of the NMDA receptor and neoplasm of the brain (Markert *et al.*, 2001). In our study, no significant differences in basal expression levels of the NMDA receptor mRNA expression were observed among the various HOSE and OVCA cell lines. However, the nonmalignant and malignant cell lines exhibited opposite responses to FSH with regard to NMDA receptor expression, which was upregulated in HOSE cells and downregulated in OVCA cells. The precise biological significance of the differential response

is unclear. Wang *et al.* (2000) suggest that upregulation of NMDA receptors in neurons may increase apoptosis. It remains to be ascertained whether FSH-induced reduction in NMDA receptor expression in OVCA cells may affect cell survival by altering recognition to apoptotic-inducing signals.

RNA helicases are present in all organisms, and over 100 such proteins are reported in sequence database. They can unwind short duplex RNAs in an ATP-dependent manner (Schmid and Linder, 1992; Fuller-Pace, 1994; Venema *et al.*, 1997). ATP-dependent helicases are implicated in pre-mRNA splicing, ribosomal biogenesis, RNA export from the nucleus, translation initiation, and mRNA decay (Fuller-Pace, 1994; Venema *et al.*, 1997). In the process of ribosomal biogenesis, RNA helicases play a crucial role both in structural rearrangements and as directional forces during the process, contributing significantly to the efficiency, accuracy, and fidelity of cellular processes. The present study revealed no differences in ATP-dependent RNA helicase expression between normal HOSE and OVCA cells; however, FSH reduced the expression of ATP-dependent RNA helicase in OVCA cells and enhanced expression of this gene in HOSE cells. The biological significance of this difference in the process of ovarian carcinogenesis remains to be resolved by future studies.

Lastly, two genes, restin and neogenin, were found to be overexpressed in OVCA cells when compared to HOSE cells. Again, expression of these two genes is upregulated in HOSE cells and downregulated in OVCA cells following FSH treatment. Neogenin is mapped to chromosome 15 in the band 15q22.3-q23 and encodes a 1461-amino-acid protein with 50% amino-acid identity to the human tumor suppressor molecule deleted in colon cancer (DCC) (Vielmetter *et al.*, 1994; Meyerhardt *et al.*, 1997). Neogenin is believed to be involved in tissue growth regulation, cell-cell recognition, cellular transition from proliferation to terminal differentiation, and cell migration (Vielmetter *et al.*, 1994). Although neogenin expression has been detected in many adult tissues, there is no information about the status of neogenin expression in the human ovary. Neogenin is present in tissues where active growth takes place, and overexpression of neogenin has been observed in a wide variety of human cancers such as breast, pancreas, brain, cervix, colon, and rectum (Vielmetter *et al.*, 1994; Meyerhardt *et al.*, 1997). Our finding that neogenin is upregulated in OVCA cells is in agreement with other studies suggesting that it plays a role in cell proliferation (Vielmetter *et al.*, 1994; Meyerhardt *et al.*, 1997). However, we here report for the first time that downregulation of neogenin expression by an antisense ODN strategy induces dramatic apoptosis in OVCA cell culture, suggesting that neogenin protein is necessary for the survival of OC cells. Collectively, our data demonstrate that normal HOSE does not require neogenin for proliferation/survival but that OC cells do. This functional dichotomy of neogenin between normal and malignant HOSE cells could be a result of changes brought about during neoplastic transforma-

tion of normal HOSE cells to malignant OVCA cells. In normal HOSE cells it appears that neogenin has functions other than cell proliferation/survival, since its expression is upregulated by FSH. Neogenin may serve as a tumor promoter in cancer cells by increasing cell proliferation/survival but, interestingly, its expression is downregulated by FSH. The later observation supports a part of epidemiological data implicating FSH as a protective agent against ovarian cancer.

Restin, a 160-kDa protein possessing features characteristic of intermediate filament protein, is mapped to chromosomal region 12q24.31-q24.33 (Hilliker *et al.*, 1994). It facilitates the microtubule-dependent transport of endosomal vesicles (Poerre *et al.*, 1992; Rickard and Kreis, 1996). It has also been localized to prometaphase kinetochores and appears to play a role in correct spindle assembly and normal chromosomal segregation (Dujardin *et al.*, 1998). It is overexpressed in malignant cells of Hodgkin's disease and large-cell lymphoma (Bilbe *et al.*, 1992; Hilliker *et al.*, 1994), consistent with our finding of higher expression of restin in OVCA cells compared to normal HOSE cells. FSH induces upregulation of restin in HOSE cells, which may disrupt spindle assembly and chromosomal segregation, leading to genetic instability. In this regard, the hormone may promote neoplastic transformation of normal ovarian epithelial cells. Moreover, as shown in the present study, restin is overexpressed in OVCA cells as compared with HOSE cells. The ramification of restin overexpression in OVCA cells is currently unknown, but our results clearly show that it promotes cell proliferation/survival in a manner similar to neogenin. Ironically, FSH has been shown to downregulate restin expression specifically in OVCA cells. If this scenario holds true for OCs, it would support a protective role of FSH by downregulating cell growth-promoting factors in ovarian neoplasms. In short, similar to neogenin, restin appears to play a different role in normal HOSE cells than it does in cancerous OVCA cells. Likewise, its upregulation by FSH in HOSE cells and inhibition by the hormone in OVCA may explain some of the controversial claims in the literature that FSH both promotes and protects against ovarian carcinogenesis.

One of the most interesting findings of this study is that FSH downregulates about 92% of the genes in OVCA cells and either upregulates or downregulates about 50% of the genes in HOSE cells. (The complete gene list is available at <http://users.umassmed.edu/Shukmei.ho>.) The precise mechanism by which FSH regulates an almost completely different set of genes following cellular transformation is unclear. However, several scenarios have been proposed. FSH binds to specific G-protein-coupled receptor and stimulates adenylate cyclase to produce cAMP. The activation of cAMP protein kinase PKA results in phosphorylation of certain transcription factors, which bind to cAMP responsive elements (CRE) to induce or repress the expression of FSH-responsive genes (Mukherjee *et al.*, 1998). These cAMP responsive factors, cAMP binding protein (CREB), and CRE-modulatory protein (CREM) can act as cAMP-regulated stimulators or

repressors of gene transcription. Inducible cAMP early repressor (ICER, a member of the CREM family) is a powerful repressor of cAMP-mediated transactivation (Sassone-Corsi, 1998). Aberrant expression of CREB and/or ICER may explain why FSH would regulate completely different sets of genes in a negative manner in cancer cells.

Although the present study identified FSH-regulated genes in HOSE or OVCA cell lines, and a specific PKA inhibitor was shown to block the expression of identified genes, it remains possible that some of these genes are regulated by FSH only indirectly. This possibility springs from the fact that gene profiling was conducted after cell cultures were treated for 5 days with FSH. During this relatively long period of treatment, many secondary events could have occurred, making it likely that a number of differentially expressed genes may be the results of downstream effects of the hormonal treatment.

Finally, it is of significance that we here report FSH-induced downregulation of a plethora of genes that are vital for cell functions, such as those that are involved in RNA metabolism, regulation of intracellular calcium signaling, synthesis, phosphorylation, folding and degradation of proteins, protection against harmful physiological stresses, and recognition of apoptotic signals, as well as those exhibiting tumor suppressor or oncogenic functions. To our knowledge, none of the genes for which we found downregulation by FSH have been previously reported in normal or malignant human ovarian epithelial cells, and no information is available regarding their regulation by FSH. Microarray has proven to be an effective tool for the discovery of new genes regulated by a specific hormone and for discovery of new genes involved in carcinogenesis and tumor progression. Hence, findings from this study are the first to corroborate FSH as an important regulator of OVCA cell functions and suggest that FSH may be a modulator for ovarian cancer progression, since the hormone 'switches' its regulation to a new set of genes not normally under its influence in normal HOSE cells.

Materials and methods

Cell cultures and cell lines

The origin and culture conditions of HOSE and OVCA cell lines have been previously described (Lau et al., 1999; Rauh-Adelmann et al., 2000; Syed et al., 2001; Syed et al., 2002). In short, three HOSE cell lines, HOSE 642, HOSE 6-8, and HOSE 12-12, were derived from normal ovaries obtained from women with noncancer gynecologic indications, specifically, a 46-year-old normal individual, a 48-year-old patient with ovarian inclusion cyst in one of the ovaries, and a 39-year-old patient with ovarian stromal hyperplasia, respectively. Primary cultures were established from surface scrapings of these normal ovaries and immortalized with human papillomavirus E6 and E7 (Tsao et al., 1995). The immortalized cell lines exhibited uniform epithelial-like morphology; immunopositivity for cytokeratins K7, K8, K18, and K19, and immunonegativity for vimentin. The immortalized HOSE cell lines were shown to be nontumorigenic in nude mice and expressed no

CA-125 (Taso et al., 1995). OVCA 420, OVCA 429, and OVCA 432 were established cell lines derived from patients with late-stage serous ovarian adenocarcinomas, as described by Bast et al. (1981). These cell lines were cultured at 37°C in a humidified atmosphere of 5% CO₂/95% air in a 1:1 mixture of medium 199/MCDB 105 (Sigma, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma), penicillin (100 U/ml; Sigma), and streptomycin (100 µg/ml; Sigma).

Hormone treatment of HOSE and OVCA cell lines

Cell lines cultured in medium 199/MCDB 105 were harvested when 80% confluent and washed in phosphate-buffered saline (PBS), then 2 x 10⁵ cells were seeded per T-25 flask (Falcon, Becton Dickinson Labware, Bedford, MA, USA culture area = 25 cm²) and allowed to attach for 24 h. After 48 h, later, medium was replaced with medium 199/MCDB 105 containing charcoal-stripped FBS supplemented with 10⁻⁶ M FSH (Calbiochem, San Diego, CA, USA) (treated) or aqueous saline vehicle (untreated). Cell cultures were subsequently treated daily for 5 days with and without the hormone. The treatment time was chosen based on our previous study (Syed et al., 2001), in which maximum proliferation of HOSE and OVCA cells was achieved after 5 days treatment of cells with FSH. At the end of the treatment period, cells were harvested from FSH-treated and untreated cultures for total RNA isolation. RNA preparations from this set of cultures were used for cDNA microarray analyses.

For *post hoc* real-time RT-PCR analyses, a separate set of cultures was exposed to 10⁻⁸ M FSH in the presence or absence of a protein kinase A (PKA) inhibitor. A dose of 10⁻⁸ M of the hormone was chosen because it fell at the midpoint of the dose-response curves previously reported for FSH-induced cell proliferation for these cell lines (Syed et al., 2001). The protein kinase A selective inhibitor H-89 (*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide), HCl (Calbiochem), with an inhibition constant of 0.048 µM, was added at a final concentration of 10⁻⁵ M to half of the cultures 30 min before treatment with 10⁻⁸ M FSH. We have previously demonstrated that H-89 at this concentration effectively blocked the action of FSH and LH in HOSE/OVCA cells (Syed et al., 2001). After 5 days of daily treatment with FSH with or without H-89, cells were collected and total RNA was extracted. This experiment was repeated twice.

RNA isolation and microarray experiments

Total RNA was isolated from FSH-treated and untreated cultures using the TRI reagent (Sigma) according to the manufacturer's protocol. RNA integrity was validated according to previously described protocols (Syed et al., 2001, 2002). The MICROMAX™ Tyramide Signal Amplification (TSA) labeling and detection kit (NEN, Life Science Products, Inc., Boston, MA, USA) was used according to the protocol provided by the manufacturer. The TSA labeling protocol was chosen because it is a posthybridization amplification system that permits detection of differential transcript expression in samples with relatively small amounts of total RNA. Briefly, 4 µg of total RNA from the untreated and FSH-treated HOSE/OVCA cell lines was subjected to cDNA synthesis using biotinylated and fluorescein (FL)-labeled nucleotides, respectively. Equal quantities of two cDNA probes were mixed and hybridized on a cDNAs microarray slide in a Corning microarray hybridization chamber (Corning Inc., Corning, NY, USA) at 65°C overnight. The hybridized slides were washed under stringent conditions. The FL-labeled probes on

the slide were recognized by an anti-FL antibody that was conjugated to horseradish peroxidase (HRP). The enzyme catalyzed the deposition of cyanine 3 (Cy3)-labeled tyramide on the labeled cDNA. After the inactivation of residual HRP from the first reaction, streptavidin-linked HRP was used to deposit Cyanine 5 (Cy5) tyramide to the biotin-labeled cDNA probe on the slide. The slides were washed extensively and air-dried. The hybridized microarray slides were scanned using a ScanArray™ confocal laser scanner (GSI Lumonics, Inc., Watertown, CA, USA) at 10 μ m pixel resolution for Cy3 and Cy5 signal detection. ImaGene analysis software (BioDiscovery, Inc., Los Angeles, CA, USA) was used to quantify Cy3 and Cy5 signals on each spot. For each spot, the average intensity of all pixels within intensity values falling in the range of 50–95% of maximal intensity was calculated (the 'signal intensity'). The 'background signal' was calculated from the average intensity of pixels within 5–15% of maximal intensity in the background ring immediately outside the spot of interest on the cDNA microarray. The 'corrected signal intensity' for each spot is calculated as the 'signal intensity' minus the 'background signal'. The complete list of the 2400 genes that were spotted as cDNAs on the MICROMAX microarray™ can be found at <http://www.nen.com/products/gene-list5.txt>.

Identification of differentially expressed genes using *t*-statistics

Before data from the same slide or from different slides could be compared, it was necessary to normalize the data (i.e., remove or adjust for intrinsic and extrinsic variations) (Yang *et al.*, 2000). For within-slide normalization, the global median normalization method was used in this study. It assumes that the red and green intensities are related by a constant factor, that is, $R = kG$, and the center of the distribution of log ratios is shifted to zero: thus

$$\log 2R/G \rightarrow \log 2R/G - c = \log 2R/(kG)$$

For normalization of a particular gene set, we used the median of the intensity log ratios M as the location parameter $c = \log 2k$. After within-slide normalization, all normalized log ratios were centered around zero. In order to compare expression levels across different slides, we applied the same normalization principles used for within-slide normalization. In this study, we chose to use all genes for normalization instead of using the microarray housekeeping gene pool for normalization because, as elaborated by Yang *et al.* (2000), this approach offers the most stability in terms of estimating space- and intensity-dependent trends in the log ratios.

Differentially expressed FSH-regulated genes were then identified by computing two-sample Welch *t*-statistics as previously described (Callow *et al.*, 2000). Briefly, the normalized DNA microarray data of gene expression profiles for untreated ($n_1 = 3$) and FSH-treated HOSE/OVCA cells ($n_2 = 3$) were presented as a matrix in which columns correspond to n_1 and n_2 and rows correspond to 2400 genes (cDNA spots on the MICROMAX™ microarray slide). Null hypothesis H_0 of equal mean expression of untreated and FSH-treated HOSE/OVCA cells for gene j ($j = 1, 2, 3, \dots, 2400$) was tested. For gene j , the *t*-value was calculated as follows:

$$t_j = \frac{\chi_{1j} - \chi_{2j}}{\sqrt{s_{1j}^2/n_1 + s_{2j}^2/n_2}}$$

where χ_{1j} and χ_{2j} denote the mean expression of gene j in untreated (n_1) and FSH-treated (n_2) HOSE/OVCA cells lines, respectively. The s_{1j}^2 and s_{2j}^2 denote the variances of gene j in untreated and FSH-treated HOSE/OVCA cell lines, respec-

tively. The *t*-value for each gene was calculated; a large absolute *t*-value suggests that the corresponding gene shows potentially significant differences in expression levels between the untreated cell lines and the FSH-treated cell lines. Evidence against the null hypothesis (the *P*-value) for each gene was also obtained. Genes with an absolute *t*-value > 2 and a *P*-value < 0.05 were recognized as genes whose expression was significantly altered by FSH in all three cell lines of the group (HOSE or OVCA).

Post hoc analyses using real-time RT-PCR quantification of mRNA expression levels

After DNase I treatment, 1 μ g of total RNA preparation obtained from a HOSE or OVCA cell line, treated with FSH or untreated, was used to generate the cDNA. To quantify and demonstrate the integrity of the isolated RNA, real-time RT-PCR analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out using mRNA-specific primers. The SYBER Green-iCycler (BioRad, Hercules, CA, USA) detection system was used to assess, via real-time RNA-PCR, the amount of targeted transcript present in each sample. Briefly, 2 μ l of cDNA derived from reverse transcription of a RNA sample was amplified in triplicate in a 25 μ l reaction mixture containing 12.5 μ l of 2 \times SYBER Green Master Mix and 10 pmol of each primer. Each PCR reaction was optimized to ensure that a single PCR product of the appropriate size (50–200 bp) was amplified and that no products corresponding to genomic DNA amplification or primer-dimer pairs were present. The following PCR cycling conditions were performed for the generation of all amplicons: 10 min at 95°C, 50 cycles for 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. PCRs for all templates were performed in triplicate in a 96-well plate format, along with positive and negative controls as well as a calibrator sample. The data shown are averages of two separate experiments with triplicate real-time RNA-PCR performed on each sample and are represented as means \pm s.e.m.s.

Transfection of HOSE/OVCA cells with gene-specific antisense oligonucleotides (ODNs)

Phosphothioate-modified antisense oligonucleotides (ODNs) for rap1GAP, neogenin, and restin were designed to have complementary binding to the translation start sites of the targeted mRNAs (antisense and sense ODNs for each mRNA from MWG Biotech, High Point, NC, USA). The sequence and the modified bases were as follows: rap1GAP antisense ODN is 5'-G*C*A*TCTTCT CAATCAT*C*T *C-3' and sense ODN is 5'-G*A*G*ATGATTGAGA*T*G*C-3', neogenin antisense ODN is 5'-G*C*C*ATCTCTTCCCCG*A*-G*A-3' and sense ODN is 5'-T*C*T* CGG GGA AGA GAT* G*G*C-3', and restin antisense ODN is 5'-G*G*C* TTT AGC ATA CTC A*T*T*T-3' and sense ODN is 5'-A*A*A* TGA GTA TGC TAA A*G*C*C-3'. Lyophilized ODNs were reconstituted in sterile distilled water and stored at -20°C. The uptake of phosphothioate ODNs by cells was facilitated using LipofectAMINE PLUS (Life Technologies, Inc, Rockville, MD, USA). Approximately, 60–80% confluent cells were transfected with antisense or sense ODNs (1.25, 2.5, and 5 μ M) in serum-free medium for 3 h at 37°C, incubated with complete medium for 24 h, and treatment was repeated once before termination of cultures for RNA extraction. For each ODN, triplicate cultures were transfected for each of the three doses. Cell proliferation was measured either by cytometer counting or by spectrophotometry. In some cases, the cells were harvested by scraping, washed with PBS, pelleted

and counted using a hemocytometer. In other cases, proliferation was measured by using Promega's Cell Titer Aqueous assay, in which viable cells convert MTS tetrazolium into a formazan-colored product. After the incubation period, 400 μ l of the MTS/MPS/medium solution was added to each well and plates were incubated for 4 h in a humidified atmosphere. To determine the cell number in each well at the end of the incubation period, the amount of formazan formed was measured as absorbance at 490 nm in a spectrophotometer. Assays were performed in triplicate to generate mean values for control and each treatment group. Cell number, as measured by the rate of formazan formation in control wells with untreated cells, was considered to be 100%. Data points in all figures are group mean values \pm standard deviations from three separate experiments.

Semiquantitative reverse transcription (RT)-PCR

Relative expression levels of rap1GAP, neogenin, restin, and proliferating cell nuclear antigen (PCNA) transcripts were determined by RT-PCR 48 h after the second transfection of ODNs. The data shown are means of triplicate cultures for each ODN at the three concentrations and are represented as means \pm s.e.m.s.

Caspase activity assay

After ODN treatment, the detached cells were harvested by centrifugation at 400 g for 10 min at room temperature, washed once with PBS, and the cell pellets were held on ice. The adherent cells were washed twice with PBS, scraped off in 50 μ l of ice-cold lysis buffer included in the caspase assay kits (Clontech Laboratories, Palo Alto, CA, USA), and pooled with the detached cells. The cell lysates were microcentrifuged at maximum speed for 3 min at 4°C to precipitate cellular debris. Supernatants were transferred to new tubes. A volume of 50 μ l of the supernatant was added to each well. After 50 μ l 2 \times reaction buffer/DTT Mix and 5 μ l of 1 mM caspase substrate (DEVD-pNA; 50 μ M final concentration) were added

to each reaction, the mixture was incubated at 37°C for 1 h. Absorbance was recorded on a plate reader at 405 nm. The net increase of absorbance was indicative of enzyme activity.

Cell invasion assay

Clontech Biocoat™ Matrigel™ Invasion chambers were used to assess the invasive property of OVCA cells *in vitro*. Matrigel chambers were rehydrated at 37°C for 2 h. Control cells or antisense rap1GAP ODN-treated OVCA cells (2.5×10^4 cells, OVCA 420, OVCA 429, and OVCA 432) as described above were cultured in Matrigel Invasion Chambers for 22 h in humidified atmosphere at 37°C. Serum was used as the chemoattractant. We used ovarian cancer cell line SKOV3 as a positive control. After 22 h, noninvading cells were removed from the upper surface of the membrane by scrubbing. The cells on the lower surface of the membrane were fixed for 2 min in 100% methanol and stained in 1% toluidine blue in 1% sodium borate for 2 min. Excess stain was removed by rinsing the insert with water. Membrane was removed from the insert and placed on a microscopic slide on which a small drop of immersion oil had been placed. The cover slip was placed on the slide and cells were counted. Percent invasion was determined by dividing the mean number of antisense-treated cells invading through Matrigel insert membrane by the mean number of control cells migrating through control Matrigel insert membrane multiplied by 100. The assay was run in triplicate.

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